GLOMERULAR PERMEABILITY TO MACROMOLECULES

by

PAMELA R. MACLEAN, B.A.

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The glomeruli of the kidney were first seen in 1666 by Malpighi, who thought that they were "glands" which secreted urine. It was not until 1842 that Bowman rediscovered them and considered them to be a vital part of the normal functioning kidney. The concept of renal function as filtration by the glomeruli, followed by conservation of water and essential solutes by the tubules, was to remain a hypothesis, however, until the twentieth century.

Experimental work, in health and disease, and in man and animals, has now amply confirmed Bowman's original ideas of filtration and reabsorption. With the introduction of new and sensitive chemical techniques knowledge of the functioning of the normal and abnormal kidney has rapidly accumulated. Studies of the renal structure were for some time limited to the light microscopic examination of renal ~~biopsy specimens~~ tissue obtained post mortem. More recently development of percutaneous renal biopsy and the application of electron microscopy to biological material have enabled studies of the fine ultrastructure of the kidney to be carried out, and the pathology of the disease processes to be studied during the course of the illness.

It has been known for over a century that in renal disease macromolecules escape into the urine from the plasma. Histological studies have shown that the primary renal lesion is generally to be seen in the glomerulus. The inference that the diseased glomerulus ceases to be an efficient filter has been confirmed both directly and indirectly by several workers. However, the relationship between the functional and ultrastructural aspects of the glomerulus is still not fully understood. This study has been undertaken in an attempt to define more closely both normal and abnormal glomerular permeability to macromolecules, and if possible to relate the results to biochemical changes and to ultrastructural abnormalities as seen by light and electron microscopy.

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Since glomerular structure and function are directly linked to problems of glomerular permeability, a brief description of the light and electron microscopical appearances of glomeruli is given first and this is followed by a description of glomerular function. Function in relation to ultrastructure is then discussed. Both normal and abnormal glomeruli are considered.

In the second section an account is given of the development of theories concerning the permeability of the normal glomerulus. This is followed by a consideration of present knowledge of the subject and some of the problems it raises. Glomerular permeability in renal disease is then discussed, and the attempts that have been made to define the abnormality in functional terms are described.

Finally the aims of the present research are described and discussed.

1.1. GLOMERULAR ULTRASTRUCTURE AND FUNCTION

Glomerular ultrastructure

The glomerulus as seen by light microscopy was described by Vimtrup (1928) and McGregor (1929a) and consists of a tuft of capillary vessels inserted in the course of an arteriole. The capillary loops lie within a space, which is bounded by Bowman's capsule. The glomerular capillaries are lined internally with endothelium, which is separated from the external epithelium by basement membrane.

Electron microscopy has revealed several unsuspected and interesting features of the glomerulus. One of the first descriptions of mammalian renal tissue, as seen by the electron microscope, was published in 1950 (Pease and Baker, 1950). Since then numerous reports have confirmed and extended the early results. The subject is well reviewed by Mueller (1958).

The endothelial cells are three to four times as numerous as the epithelial cells and form a fenestrated layer of cytoplasm of 1000-2000 Å. The pores measure about 1000 Å in diameter and allow direct contact between the basement membrane and the contents of the capillary lumen.

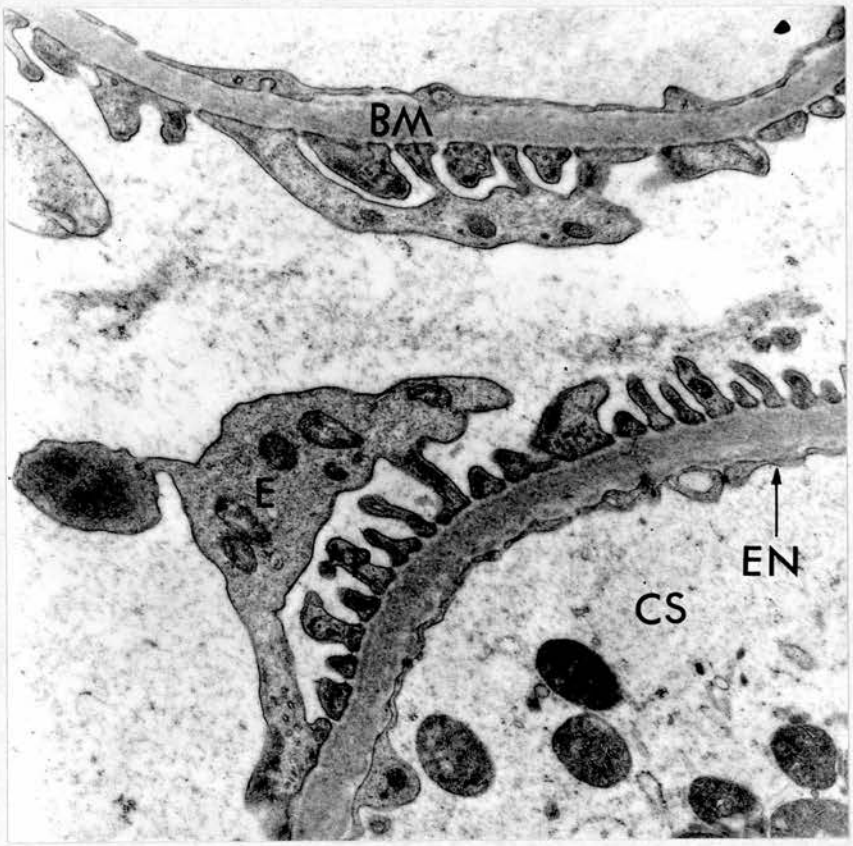
The epithelial cells have a more complex structure than the endothelial cells with numerous vacuoles, vesicles, a Golgi zone and many mitochondria, which all tend to be located towards the centre of the cell. The peripheral structure consists of cytoplasmic processes called pedicels, which lie in contact with the basement membrane. The pedicels are separated from one another by "slit pores" of about 1000 Å and across each pore a fine membrane is stretched.

The basement membrane, which lies between the endothelium and epithelium, is a relatively homogeneous layer, measuring approximately 2500-3500 Å. It is composed of glycoprotein and mucopolysaccharide. After staining

Fig. 1. A normal glomerulus.

The basement membrane is of uniform thickness. Each capillary loop has an endothelial lining. Discrete pedicels pass onto the basement membrane from the epithelial cells.

Magnification x 15,000



BM	basement membrane
E	epithelial cell
CS	capillary space
EN	endothelial cell
P	pedicel

with heavy metals a fine fibrillar structure may be visible, the fibrils being 30-40 Å in diameter.

Fig. 1 shows a normal glomerulus as seen by the electron microscope.

In glomerular disease abnormalities are almost always seen in both the endothelium and epithelium and very often the basement membrane is also affected (Farqhuar, 1959). In most cases the changes can be seen on light microscopy but in a few cases the abnormalities can only be seen on electron microscopy (Kark et al., 1958).

The structural changes are many and varied, depending on the type and extent of the renal disease, but in general the picture is as follows: (a) the epithelial cells show striking changes with fusion of the foot processes into a continuous smearing of cytoplasm along the external surface of the basement membrane; (b) proliferation of endothelial cells is apparent, there is broadening of the endothelial cytoplasm lining the capillary and there is a decrease in the number and regularity of the fenestrae or endothelial slit pores; (c) the basement membrane is often irregularly thickened and may contain "deposits" of materials which stain with heavy metals. Spiro (1959) considered the basement membrane was always abnormal, but other workers have described it as normal in a small percentage of cases (Kark et al., 1958).

Glomerular function

The concept of filtration of plasma by the glomerulus, producing a protein-free fluid, was elaborated by Cushny (1926). Indirect evidence of ultrafiltration, or some closely related process, by the glomeruli was obtained from observations that urine ceased to flow if pressure across the glomerular membrane was grossly diminished, either by lowering the blood pressure in the renal arteries or by obstructing the ureters (Robinson, 1962).

Studies of glomerular function are complicated by the fact that the glomerular filtrate is modified by the tubules before it becomes urine. Direct glomerular puncture is difficult to perform and is obviously restricted to animal studies, but the limited application of this technique has confirmed that glomerular fluid is similar in composition to an ultrafiltrate of plasma. Wearn and Richards (1924) showed that the glomerular filtrate of the frog and Necturus contained less than 50 mg. per 100 ml. of protein. By suppressing the metabolic activity of tubular epithelial cells by cyanide, or by a reduction in temperature, many workers have found that the urine (which then virtually represents the glomerular filtrate) resembles an ultrafiltrate of plasma. (Bayliss and Lundsgaard, 1932; Bickford and Winton, 1937; Nicolson, 1949).

The concept of a protein-free glomerular filtrate was first challenged by de Haan (1922) who found protein-bound dyes in the urine after intravenous administration. Some twenty years later the early view of an entirely protein-free glomerular fluid was modified following the work of Walker et al. (1941) and Bott and Richards (1941). The former workers found small concentrations of protein in some of their samples of glomerular fluid from the rat and guinea pig, and the latter showed that perfusion of kidneys of Necturus could result in the presence of protein in the glomerular filtrate. Perfusion of rabbit kidneys with ice cold serum resulted in a "urine" containing 15-22 mg. protein per 100 ml. (Dock, 1942); the serum was cooled in order to paralyse the tubular function. Smetana (1946) found considerable concentration of protein bound dyes in the proximal tubules of several mammalian species and considered that this was due to normal glomerular filtration of protein. More recently Dirks, Clapp and Berliner (1964) examined the protein concentration in the proximal tubule of the dog, and

found 18 out of 38 samples to contain protein concentrations of 2 - 8 mg. per 100 ml.

Possible objections to this experimental evidence can be made, on the grounds that puncture of protein-containing tissue could invalidate the finding of small quantities of protein in the glomerular filtrate. Also, some of the work demonstrating glomerular filtration of protein has been carried out in the rat, an animal with a physiological proteinuria (Gilson, 1949). One group consider this proteinuria is caused by a tubular secretion of protein derived from the plasma (Shuster, Jones and Flynn, 1962). However the fact that many workers have demonstrated higher protein concentrations in the tubules than in the urine suggests that there is active tubular reabsorption of protein, making the concept of secretion of protein unnecessary and unlikely (Sellers et al., 1954; Spector, 1954a, Mendel, 1961). In addition the studies of Farqhuar et al. (1961) demonstrated that a small percentage of ferritin molecules can cross the glomerular capillary wall of the rat, suggesting that the glomerulus is the source of urinary protein. It is now almost universally accepted that the glomerulus is not a perfect filter, the normal glomerular filtrate containing a small quantity of protein, which is almost entirely reabsorbed by the tubules. The concept of tubular reabsorption of protein will be discussed later (1.2).

The efficiency of the filtering capacity of the glomerulus is usually estimated by measuring the concentration, in the plasma and the urine, of a substance which passes so freely through the glomerulus that the concentration in the glomerular fluid is the same as that in the plasma. This substance must be neither reabsorbed nor secreted by the tubules. The urinary concentration, in mg. per minute, represents the concentration in the glomerular filtrate and the term UV/P where U = urinary concentration

in mg./ml., V = urine flow in ml./min., and P = plasma concentration in mg./ml. is called the glomerular filtration rate (G.F.R.). In theory inulin is the most suitable compound, but an intravenous injection and many blood samples are required for an inulin clearance. In practice the clearance of endogenous creatinine provides a sufficiently accurate measurement of G.F.R. Although some creatinine is actively secreted by the tubules this effect is counterbalanced by the plasma chromogens, which give the same orange colour with picric acid as creatinine. Some authors have expressed doubts about the value of creatinine clearances in proteinuria and uremia (Berlyne et al. 1964), but others have concluded that creatinine clearance is a good index of G.F.R. in chronic renal disease (Tobias, McLaughlin and Hopper, 1962). The clearance of radioactive vitamin B_{12} has recently been described as an accurate measurement of G.F.R. However, a very recent paper concludes that endogenous creatinine clearance is a more valuable estimate of G.F.R., even in renal disease (Malamos et al., 1966). Glomerular filtration rates in healthy human subjects average 125 ml./min. (Robinson, 1962).

In renal disease with glomerular impairment the G.F.R. may be marginally or severely reduced, the whole range of values from over 100 ml./min.down to 1 or 2 ml./min. being encountered. Impaired glomerular function is also indicated by an accumulation, in the plasma, of substances such as urea and creatinine, which are normally excreted in the urine. The blood urea generally shows a rise prior to the rise in serum creatinine. Blood urea levels of over 40 mg./ml. are usually associated with a reduced G.F.R. and an ultrastructural renal lesion.

The presence in the urine of protein, casts, or red cells generally implies renal involvement, which is often of glomerular origin. The

excretion of macromolecules in renal disease is discussed later (1.2). Along with the proteinuria there may be a reduction in some of the plasma proteins, notably albumin, and a rise in the plasma level of α_2-globulin.

Glomerular function in terms of ultrastructure

Kurtz and McManus (1959) and Mueller (1958) have reviewed some of the opinions expressed concerning the function of the various structural elements of the glomerular capillary. The function of the epithelial and endothelial cells is not known, although it is suggested that they have a low metabolic activity (Montgomery, 1965). They may both be concerned with phagocytosis of particulate materials. Farqhuar et al. (1961) suggested that the endothelium acts as a possible valve by varying the number and distribution of fenestrae, thereby controlling the area of basement membrane exposed to the plasma. These authors thought the epithelium might act as a monitor for the basement membrane, regulating the passage of plasma proteins. The fact that smearing of the epithelial cells is almost always the first abnormality to be seen during proteinuria supports this view.

The majority of electron microscopists have assumed that the basement membrane acts as the main filter, since it is the only continuous structure between the plasma and the urinary space (Mueller, 1958; Farqhuar, 1959). According to Hall (1957), however, the slit pores between the epithelial foot processes allow the passage of small molecules, but restrict the passage of proteins. This hypothesis is difficult to reconcile with the measurement of the diameter of the slit pores which is approximately 1000 Å (Vernier, 1961). Farqhuar and associates have provided good evidence that the basement membrane acts as the principal filtration barrier (Farqhuar and Palade, 1960; Farqhuar et al., 1961). These papers describe the glomerular transport of ferritin in normal and nephrotic rats. In the normal rat ferritin molecules

were observed to enter the endothelium and form an accumulation against the basement membrane, with only a few molecules penetrating the basement membrane and entering the epithelium. In the nephrotic rats transport of ferritin was greatly increased and more molecules were seen in the endothelium, basement membrane and epithelium. The basement membrane therefore acts as the filtration barrier, which is not readily permeable to molecules of about 100 Å in health, but is significantly more permeable in renal disease.

The mechanism whereby the glomerular capillary transports material is uncertain. The main barrier, the basement membrane, has no visible pores or channels and ferritin tracer molecules do not appear to follow any preferred pathways (Farqhuar et al., 1961). Lambert and Gregoire (1955) argue that diffusion is three times as important as filtration in the passage of proteins, but Pappenheimer (1953) considers filtration to be a much more important process, where large molecules are concerned. A third means whereby larger molecules can cross the capillary wall was suggested by Mayerson et al., (1960). These authors described the process as cytopempsis: materials transported from one surface of a cell to another in minute vesicles. Cytopempsis may well occur in the epithelium.

The overall transport mechanism is certainly a physico-chemical one which tends to retain molecules beyond a certain size. There is no indication that there is any type of selection except a dimensional one, although the extent to which lipid solubility, charge and shape may play a part is quite unknown. Gardner (1961) demonstrated changes in protein excretion occuring with changes in pH, although his conclusions that glomerular permeability was altering are not confirmed by the experimental evidence.

In glomerular diseases the structural changes which are seen are

difficult to relate to the functional changes. Evidence for the glomerular origin of proteinuria is discussed later (1.2). Immunological evidence suggests that the basement membrane, the probable site of filtration, is the site of the primary lesion (Stebley and Lepper, 1961; Andres et al., 1962) and, although the basement membrane is usually irregularly thickened, no obvious pores can be seen. The epithelial and endothelial cell changes, which are often prominent, are thought to be the result rather than the cause of the proteinuria (Fisher and Hellstrom, 1962). Although a reduced glomerular filtration rate and a gross proteinuria are usually associated with glomerular changes, in some cases gross proteinuria can coexist with normal renal function and structure. The only glomerular abnormality in minimal lesion glomerulonephritis is a smearing of the epithelial pedicel structure and this has already been mentioned as a result rather than a cause of proteinuria. In these patients dramatic responses to steroids have been described with an accompanying return of pedicel structure (Folli et al., 1958; MacDonald, Lambie and Robson, 1959). An almost normal glomerular structure is also associated with postural proteinuria, but nevertheless in this condition many high molecular weight proteins escape into the urine (Rowe and Soothill, 1961b). Haematuria, which implies a gross abnormality, sometimes occurs together with minor proteinuria and minimal glomerular damage. Such paradoxical observations raise many interesting questions which have been, and still are, challenging problems.

1.2. GLOMERULAR PERMEABILITY TO MACROMOLECULES

Normal glomerular permeability to macromolecules

An evaluation of glomerular permeability to macromolecules must also take tubular reabsorption into consideration. The early work on tubular reabsorption of protein is reviewed well by Rather (1952). Studies on the excretion of haemoglobin, a good marker substance which was easily obtained pure, indicated a renal "threshold" for this protein (Lichty, Havill and Whipple, 1932) and Monke and Yuile (1940) carried out the first quantitative study of the filtration and reabsorption of a protein. The latter workers found that with plasma haemoglobin concentrations of between 250 and 1000 milligrams % the haemoglobin clearance was 3% of the creatinine clearance in normal dogs. There was a linear relationship between the plasma concentration and the rate of urinary excretion of haemoglobin. They suggested that the glomerulus permitted the passage of haemoglobin in small amounts, relative to a substance such as creatinine, which was directly dependent on the concentration in the plasma. As this passed down the tubule some was taken up by the epithelial cells, the tubular reabsorptive capacity being saturated at plasma concentrations of over 250 mg.% haemoglobin. It has since been shown that the threshold for haemoglobin depends on the linkage of haemoglobin in the plasma to the α_2-globulin, haptoglobin (Lathem, 1959). However the demonstration of the principle of tubular reabsorption is still quite valid. Waterhouse and Holler (1948) and Terry, Church and Whipple (1948) have confirmed renal thresholds for albumin and serum proteins in human subjects and in dogs. Many morphological studies have been carried out by Oliver and co-workers which amply confirm normal tubular reabsorption of protein (Oliver, 1948, 1950; Oliver, MacDowell and Lee, 1954).

Quantitative assessments of normal tubular reabsorption are difficult

to make. Sellers et al. (1954) studied tubular reabsorption in the normal rat and concluded that 5 mg. of protein per hour was reabsorbed, an amount equivalent to a daily filtration and reabsorption of 33% of the circulating plasma protein. Gregoire, Malmendier and Lambert (1958), by analogy with infusion experiments in dogs, estimated the maximum tubular reabsorptive capacity in man to be 55 mg. per minute or 80 g. per 24 hours.

Morner in 1895 claimed that the normal urine of men and women contained up to 155 mg. protein per litre. Many workers since then have given values of normal urinary protein ranging from 39 mg. per 24 hr. to 240 mg. per litre. (Rigas and Heller, 1951; Boyce, Garvey and Norfleet, 1954; McGarry, Rose and Sehon, 1955; Webb, Rose and Sehon, 1958a; Nettleship, Strother and Smith, 1963; Saifer and Gerstenfeld, 1964). The presence of small amounts of protein in the normal glomerular filtrate has been discussed earlier (1.1). If, as an estimate (based on the direct glomerular puncture experiments) the concentration of protein in the glomerular filtrate is 10 mg. per $\frac{100}{1}$ ml. then a total of 17 gm. is filtered daily. Estimates of maximal tubular reabsorptive capacity exceed this figure (Gregoire et al. 1958). However it is possible that the tubule may not be a perfect reabsorber, a situation analogous to the imperfect filtration mechanism of the glomerulus. If the reabsorptive mechanism is not 100% efficient small quantities of protein could then escape into the urine.

General considerations of capillary permeability to plasma proteins have been reviewed by Asscher and Jones (1965). The glomerular capillaries have been shown to be much more permeable to water and small molecules than muscle capillaries, and a specialised function has been predicted (Pappenheimer, Renkin and Borrero, 1951). Physiological data and theoretical considerations suggest the glomerular membrane contains "pores" of about 50 - 100 Å,

covering perhaps 2% of the membrane surface (Pappenheimer, 1953).

The permeability of the normal glomerulus to proteins of different sizes was first investigated by Bayliss, Kerridge and Russell (1933). These authors concluded that excretion was dependent on molecular weight and that the limiting size was probably about that of haemoglobin, 68,000. This was confirmed by Marshall and Deutsch (1950). Other electrophoretic and immunological studies, however, suggest that some larger molecular weight proteins are normally present in the urine.

Electrophoretic studies of normal urine proteins have demonstrated a pattern similar to that seen in the plasma, although the relative concentrations of the fractions are different. Concentrations of albumin are reduced and range from 23 - 39% of the total, and all the globulin fractions are raised, relative to the plasma (Rigas and Heller, 1951; Boyce et al., 1954; McGarry et al., 1955; Webb et al., 1958a). The more sensitive technique of immunoelectrophoresis has yielded interesting information. Many proteins which are immunologically similar to individual plasma proteins can be seen in the urine (Grant, 1957; Berggard, 1961a; Berggard, Cleve and Bearn, 1964; Porter, 1964; Grieble, Courcon and Grabar, 1965). Some of the urinary proteins identified were of high molecular weight, namely haptoglobin, α_2-macroglobulin, α_1-lipoprotein and γ-globulin. The total numbers of components, which react with antisera against normal human plasma, that have been identified in the urine by different workers are 10, 14 and 17 (Porter, 1964; Grieble et al., 1965; Berggard et al., 1964).

Using a sensitive immunodiffusion method Rowe and Soothill (1961a) demonstrated relatively high concentrations of large molecular weight protein in normal human urine, although the amount of total protein excreted was small. Urine:serum ratios of individual proteins were estimated and

expressed as a percentage of the albumin urine:serum ratio. Values of about 100% were obtained for siderophilin (mol. wt. 90,000), γ-globulin (mol. wt. 150,000) and ceruloplasmin (mol. wt. 150,000). This was termed an "unselective pattern" since the glomerulus was apparently acting as an unselective type of filter and allowing high molecular weight protein to pass into the glomerular filtrate. The presence of blood cells in normal urine is considered to be due to the passage of single cells through the capillary membrane (Lipmann, 1957), a view which also supports unselective normal glomerular filtration.

Although immunologically similar to plasma proteins, the normal urinary proteins could be degraded but antigenically active protein fragments of the parent molecules. Rowe and Soothill (1961a) found material of sedimentation constant of only 4S and less on ultracentrifugal analysis. In support of this there is good evidence that γ-globulin exists in a low molecular weight form in normal human urine and can cross-react with antisera to the 7S γ-globulin with a molecular weight of 150,000 (Franklin and Kunkel, 1957; Fahey, 1963; Cornillot et al., 1963). However since this small γ-globulin is also found in the plasma (Berggard, 1961b; Takahashi and Schmid, 1962) the urinary counterpart could be simply derived by rapid plasma clearance. There is conflicting evidence regarding normal urinary albumin, which has been described both as electrophoretically indistinguishable from serum albumin and as having a different mobility from serum albumin by different workers (Webb, Rose and Sehon, 1958b; Grieble et al., 1965).

The existence in normal urine of proteins which are specific to the urine, and therefore not derived from the plasma was demonstrated by Grant (1959). These proteins are probably derived from the urinary tract. The genitourinary system as a possible source of high molecular weight urinary

protein is discussed by King and Boyce (1963).

Studies of glomerular permeability to macromolecules other than protein give results which support a limiting size for filtration. Brewer (1951) examined renal clearance of low molecular weight dextran in rabbits and showed that excretion was related to molecular weight. Wallenius (1954) Arturson et al., (1964) and Arturson and Wallenius (1964a, 1964b) confirmed this and demonstrated a limiting molecular weight of 60,000 for renal clearance of dextran in human subjects. Ozawa and Yamuchi (1963) related their findings from similar experiments to an effective pore radius, which they calculated to be up to 80 Å, with a mean of 50 Å.

In summary, there are conflicting views about normal glomerular permeability. Protein studies suggest that the glomerulus acts as an unselective filter to plasma proteins, but neither the size nor the source of the protein has been adequately confirmed. In addition, the pattern of tubular reabsorption of protein in normal subjects is quite unknown. There is no evidence to suggest that it is entirely a non-selective process and, if there is some selective reabsorption, then the urinary proteins will not reflect the pattern in the glomerular filtrate, nor glomerular permeability. Dextran studies indicate that the normal glomerulus is a selective filter, but the molecular weight range studied has been much lower. Reabsorption of dextran to any extent by the tubules seems very unlikely (Arturson and Wallenius, 1954b), so that dextrans would theoretically appear to give more reliable information about normal glomerular permeability.

Glomerular permeability to macromolecules in renal disease

Proteinuria has been recognised as a cardinal feature of renal disease since Richard Bright published his observations in 1836 (Osman, 1937). Confirmation of the plasma origin of the protein was made by Berglund,

Scriver and Medes (1935), who related the depression of the plasma protein to the loss of protein into the urine; this relationship was subsequently confirmed by Blainey and Hardwicke (1957). By plasma transfer experiments Wakim and Mackenzie (1960) demonstrated that proteinuria, although of plasma origin, was not caused by a plasma abnormality, adding confirmation to the suspected renal origin.

Early reports of light microscopy studies of renal tissue in cases of proteinuria described lesions in both the tubules and the glomeruli (Bell, 1929; Wolbach and Blackfan, 1930) and some workers considered the protein was secreted into the urine by the tubules. Cushny (1926) however maintained that a glomerular lesion, visible or invisible, was responsible for the presence of an abnormal amount of protein in the urine. McGregor (1929b) supported this view and described glomerular changes in cases of proteinuria, also noting "rather insignificant changes in the basement membrane". Bell (1938) saw thickening of the basement membrane in various forms of glomerulonephritis and, like McGregor, considered proteinuria was due to an increase in glomerular capillary permeability.

Indirect evidence of the glomerular origin of proteinuria was provided by studies of glomerular and aglomerular fish (Bieter, 1931). Proteinuria could only be induced in the former. More direct evidence was presented by Dock (1942), who found increased concentrations of Evans Blue dye (which binds to albumin) in the proximal tubules and the urine of grossly proteinuric rats, when compared to normal rats. However this observation also lent support to Addis's (1949) suggestion that there was no change in glomerular permeability, but that tubular reabsorption was diminished or absent, with the result that protein appeared in the urine. More recently Freeman and Joekes (1957) have discussed the possibility that a tubular

lesion is responsible for proteinuria. This concept is a very reasonable one (Sellers et al., 1954; Gregoire et al., 1958), since maximum estimates of the amounts of protein normally filtered and reabsorbed each day are very large (41 g., ~~and~~ 80 g. ~~respectively,~~ or alternatively 33% of the circulating plasma protein).

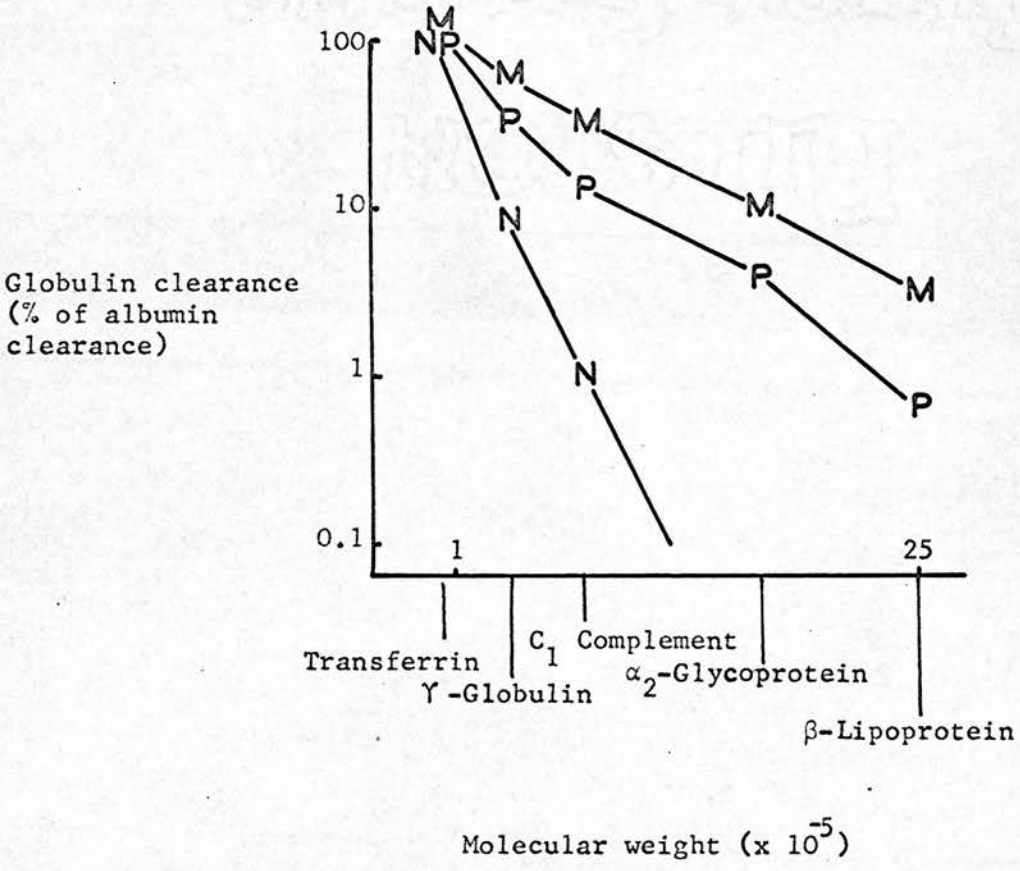
More recent work suggests proteinuria may result from a combination of altered permeability and decreased tubular reabsorption. Results of albumin infusion studies in nephrotic patients led Chinard et al. (1954) to the conclusion that "proteinuria occurs as a result of an increase in the permeability of the glomerular capillary walls to protein, rather than solely as the result of a decrease in the reabsorption of protein by the tubules". Similar studies by Gregoire et al. (1958) also indicated an increase in glomerular permeability, with a decrease in tubular reabsorptive capacity as a contributory factor. On the other hand, nephrotic rats appear to have an increased tubular reabsorption along with increased glomerular permeability (Spector, 1954a; Mendel, 1961).

The increase in glomerular permeability is now accepted as the major cause of moderate or heavy proteinuria. Many studies of proteinuria have been carried out in an attempt to relate the characteristics of the proteinuria to the type of glomerular disease. The first electrophoretic studies were described in 1940 and these were followed by many others. The findings are summarised in reviews by Sellers and Marmorston (1956) and Lewis (1963). It was concluded by Heymann, Gilkey and Lewis (1956) that a high globulin excretion had a poor prognosis, but several authors have considered electrophoretic studies could give them no guide as to the nature or prognosis of the disease (Broch and Brodwall, 1958; Wolvius and Verschure, 1957; Kasanen and Harri, 1961).

Fig. 2. Relative protein clearances in relation to molecular weight.

Immunologically determined relative protein clearances in three types of histological lesion; membranous glomerulonephritis (M), proliferative glomerulonephritis (P), and minimal lesion glomerulonephritis (N).

Taken from Blainey et al., (1960).



The molecular size of the urinary protein in renal disease was first studied by Rowe and Soothill (1957), who compared the molecular weights of the proteins of normal and nephrotic serum and nephrotic urine. The average molecular weight of the urine proteins ranged between 54,000 and 67,000, which was similar to figures obtained from serum ultrafiltrates through selective semipermeable membranes. Albumin prepared from nephrotic serum and urine had the same molecular weight as that from normal serum, a finding which was in agreement with the studies of Gitlin and Janeway (1952) and Spector (1954b). In nephrosis similar properties of serum and urinary Y-globulin (Goodman, 1961), transferrin (Neale, 1955) and orosomucoid (Hardwicke and St. Cyr, 1961) have also been described. This indicated that the protein was being excreted unchanged, although the glomerular membrane still retained a degree of selectivity. Maiorca and Scarponi (1963) concluded from their ultracentrifugal and immunological studies that fragments of high molecular weight protein occur in nephrotic urine, but they thought that the protein was degraded after, rather than before, glomerular filtration. Immunoelectrophoretic studies of urinary proteins from patients with the nephrotic syndrome have also indicated a high concentration of large molecular weight protein (Milliez, Hartmann and Lagrue, 1959; Revillard, 1964).

In 1960 Blainey et al. published a study of protein excretion in relation to molecular weight in 21 patients with the nephrotic syndrome. The individual proteins, of known molecular weight, were assayed in serum and urine by an immunodiffusion method. When the relative clearances of these individual proteins were plotted against their molecular weight on a log-log scale a linear relationship was found (Fig. 2). The slope of this line was steep when the proteinuria was of a selective type, with a relatively small amount of high molecular weight protein being cleared into the urine. Conversely

for an unselective type of proteinuria the slope of the line was flatter, with relatively more high molecular weight protein being cleared into the urine. The data suggested that the slope of the line or "selectivity" of the proteinuria was related to the disease process and also to steroid response. Hardwicke and Squire (1955), in some earlier experiments, demonstrated that tubular reabsorption of protein in nephrotic patients is non-selective, analogous to the reabsorption of amino acids. On this assumption the relative clearances obtained by Blainey et al. were related to the relative concentrations in the glomerular filtrate and hence to glomerular permeability.

There are few studies in the literature relating to abnormal glomerular permeability to other macromolecules. These publications are chiefly concerned with the presence of abnormal amounts of various enzymes in the urine in renal disease (King and Boyce, 1963). Only one author has expressed his results in terms of clearance of the enzyme and this was related to the total proteinuria (Crockson, 1961).

In summary, proteinuria is generally thought to result from increased glomerular permeability. Early electrophoretic studies of urinary protein patterns gave little information about the degree or type of lesion. More sensitive immunological techniques, which can measure the protein excreted in terms of molecular weight, have indicated that there is a relationship between the degree and type of lesion and the size distribution of the protein excreted. Tubular reabsorption of protein is assumed to be non-selective so that a direct reflection of glomerular permeability is obtained.

1.3. AIMS OF THE PRESENT RESEARCH.

The recent suggestion that glomerular permeability in disease can be defined in terms of the molecular weight of the protein excreted has raised many possibilities. At present a renal biopsy is required before a diagnosis of the degree and type of renal lesion can be made, and repeated renal biopsies are the accepted procedure in following the course of an established renal disease. If it were possible to obtain, by functional studies, some information about the renal lesion this would be a most valuable additional tool in following the progress of the disease.

Although it is recognised that glomerular permeability is usually abnormal in renal disease, the characteristics of normal glomerular permeability are in some doubt and several conflicting views have been expressed.

The present work was therefore designed to investigate more fully the renal and glomerular permeability to macromolecules both in health and disease. It was proposed initially to study the relationship between the logarithm of relative clearance and the logarithm of molecular weight, in order to confirm the linear relationship described by Blainey et al. (1960). Experiments were then designed to determine more precisely what this relationship, or selectivity, means in terms of renal and glomerular permeability and to investigate the relationship between permeability, function and ultrastructure.

Selectivity is a word which has been misused in the literature. Proteinuria has been described in terms of selectivity, but the word more accurately refers to the protein patterns of both serum and urine, and in fact indicates the ability of the kidney to act as a selective or unselective type of filter. In this study, in addition to describing proteinuria in

terms of selectivity, the word "selectivity" will be used to refer to a defined index of renal permeability, and the word "selective" will be used to refer to the kidney or the patient.

Selectivity must be calculated in terms of clearances of macromolecules since the amount of any one component appearing in the glomerular filtrate, and hence the urine, is dependent on the concentration of that component in the serum. Examination of the urine alone will give a molecular weight distribution in the urine, but without accurate knowledge of the molecular weight distribution in the serum, estimates of renal permeability in terms of molecular weight cannot be made.

Studies of both serum and urine were carried out in order to investigate selectivity in a number of ways, by using different methods and different macromolecules, by studying selectivity in different types of renal disease and normal subjects, and by comparing selectivity values with histological and other biochemical data.

2. METHODS

The methods which were used to determine the overall renal and glomerular permeability to different macromolecules in health and disease are described and evaluated. There are two main sections, which describe selectivity studies using endogenous and exogenous macromolecules.

The methods involving endogenous protein are described in Section 3. The selectivity of the proteinuria was estimated for every patient in this study and some normal subjects by an immunodiffusion technique and occasionally by immunoelectrophoresis. In some cases the quite different approach of gel filtration of Sephadex G 200 was used to study protein selectivity. The clearance of some enzymes in relation to molecular weight, and hence to selectivity, was also determined in a few instances.

The methods involving exogenous macromolecules are described in Section 4. In a few cases the selectivity of the proteinuria was investigated by immunodiffusion before, during and after a change in the serum protein distribution, effected by infusion of albumin. The renal permeability to high molecular weight dextran, in relation to molecular weight, was investigated in several patients and some normal subjects using gel filtration, and the "dextran selectivity" was determined.

In addition the total loss of protein into the urine, an important adjunct to the selectivity studies, was usually measured; the methods are described in Section 2. This section also contains details of protein estimations and protein concentration procedures which were essential to the selectivity studies.

Other additional data and relevant investigations are given in Section 1. The materials mentioned in this chapter are listed in Appendix 1, and statistical formula used in calculations are given in Appendix 2.

2.1. INTRODUCTION

Patients studied

All the patients in this study presented with proteinuria, and many with some or all of the features of the nephrotic syndrome, due to a variety of causes. In some cases the nature of the underlying disease was clear from the associated clinical features, as in lupus erythematosus, diabetes mellitus or toxaemia of pregnancy. In a few subjects the features were those of acute ischaemic renal failure or acute proliferative glomerulonephritis. In many cases however the cause of the clinical picture was obscure and the precise diagnosis was made only after renal biopsy. This group included many patients with membranous glomerulonephritis, proliferative glomerulonephritis and minimal lesion glomerulonephritis.

With very few exceptions all the patients studied had a renal biopsy performed on one or more occasions. The diagnosis was made, as far as possible, on both clinical and histological evidence. However in those subjects in whom the clinical picture did not contribute to the diagnosis, the final diagnosis was made entirely on histological grounds.

The normal subjects studied were entirely healthy, unless otherwise stated, and had no evidence of any type of renal disease.

Collection of specimens

Urine collections were normally made over a 24 hr. period. This interval has been shown to give a reliable indication of protein loss (Altman and Stellate, 1963), and is suitable for the determination of creatinine clearance estimations (de Wardener, 1963). Occasionally patients reported at an out-patient clinic with no 24 hr. urine collection and a random urine sample was taken from each of these patients for selectivity

studies only. A blood specimen was taken at some stage during the urine collection period, or within the following 8 hr. in the case of out-patients. Urine and serum or plasma were stored at 4°C until required. Sodium azide was added to the urine to give a final concentration of approximately 0.01% w/v. Where the urine required concentration this was generally carried out prior to any storage period. All the estimations were carried out, generally within one week, and always within two weeks of collecting the specimens.

In infusion experiments with exogenous macromolecules, urine collections were made over much shorter time intervals and blood specimens were taken appropriately. The details are given with the description of each experiment.

Biochemical studies

The glomerular filtration rate, as measured by the endogenous creatinine clearance, was estimated at frequent intervals for all the patients in this study. The method used was a modification of Folin and Wu's procedure, adapted for an AutoAnalyzer (Stevens et al., 1962). The coefficient of variation for the estimation of serum creatinine was 1.9% and for the estimation of urine creatinine was 4.9%.

In some cases the blood urea was also measured, either manually (Peters and Van Slyke, 1932) or by a modification of the AutoAnalyzer method of Marsh, Fingerhut and Miller (1965). The coefficients of variation of these methods were $\pm$ 12% and $\pm$ 4% respectively.

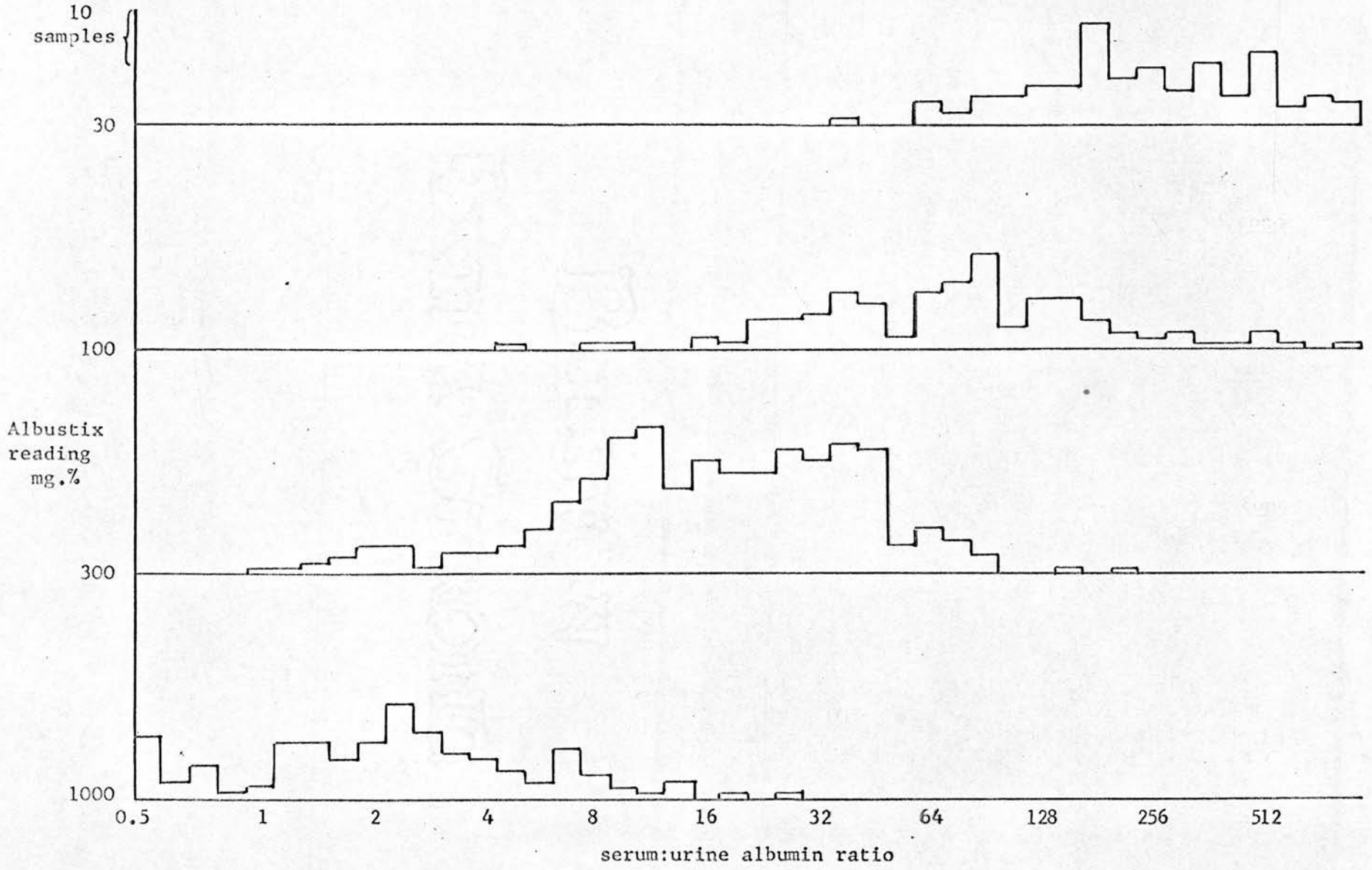
In each patient the total loss of protein in the urine was estimated by a biuret reaction. The method is described in the next section (2.2.1). In some instances the total serum protein was also estimated by a biuret reaction modified for an AutoAnalyzer (Failing, Buckley and Zak, 1960). The coefficient of variation of this method was $\pm$ 2.8%.

Electrophoresis of the serum proteins and quantitation of the different fractions was carried out by the method of Albert-Recht (1959). Coefficients of variation for the estimation of albumin, α_1-globulin, α_2-globulin, β-globulin and γ-globulin were 8.9%, 8.7%, 21%, 20% and 13% respectively.

Histological studies

Renal biopsies were obtained using a Vim Silverman type needle. Sections for light microscopy were stained with haematoxylin and eosin and with the periodic acid-Schiff stain. Additional stains were applied where indicated, for example, congo red in cases of suspected amyloid. Material for electron microscopy was fixed in 1% buffered osmium tetroxide. Tissue was generally embedded in Araldite, although in some earlier cases methacrylate was used. Sections were cut on a Porter Blum microtome with glass knives, and were viewed in an A.E.I. EM6 electron microscope. Sections from Araldite embedded tissue were stained with a combination of lead citrate and alcoholic uranyl acetate. Electron microscopy was used to study glomeruli only, and an average of five were examined from each biopsy. Where measurements of basement membrane thickness were made, electron micrographs with a magnification of 8,000 or over were used and measurements were made with a calibrated lens.

Fig. 3. Correlation of serum:urine albumin ratio determined immunologically with urinary protein concentration determined by Albustix.



2.2 ESTIMATION AND CONCENTRATION OF PROTEIN

2.2.1. ESTIMATION OF PROTEIN

Five methods are described. An approximate estimate of urinary protein concentration was obtained for the immunodiffusion studies (2.3.1) by "Albustix". Biuret procedures were used to determine more precisely the total urinary protein concentration in patients with renal disease and in normal subjects. Column chromatography effluents (2.3.3) were assayed for protein by ultraviolet adsorption and by a Folin-Ciocalteau method.

Albustix

These reagent strips are most sensitive to albumin, but will also react with other protein. They provide a good screening test for protein (Watson, 1964), although the results can be affected by high pH or high salt concentrations, and sometimes for no apparent reason (Frazer, 1958; Wills and McGowan, 1963).

The Albustix reading was satisfactorily used to determine the approximate urinary protein concentration and hence the appropriate urine dilutions for the immunodiffusion method (2.3.1). The serum:urine albumin ratio correlated fairly well with the Albustix reading (Fig. 3), in spite of the fact that the serum albumin concentration and the proportion of urinary globulin are variables which were not taken into account.

The biuret method

A simple biuret method was used to determine more precisely the concentration of urinary protein, and hence the total loss of protein (Hiller, Grief and Beckman, 1948). This was carried out in parallel with the estimation of selectivity of proteinuria for all the patients in this study.

Although the biuret produce is the most satisfactory method of quantitating proteinuria, it has been reported to give misleadingly high protein concentrations; this is probably due to lipid (Parvin, Pande and Venkitasubramanian, 1965). Lipoproteins, however, form a small proportion of urinary proteins (Kramer, Stern and Hellman, 1957; Jesting, 1964). The chromogenicity of albumin, globulins and glycoproteins is very similar with the biuret reaction, which is specific for the peptide linkage (Saifer and Gerstenfeld, 1964); different mixtures of proteins will therefore give similar results.

Precipitation prior to estimation of urinary protein is necessary, since the urine contains many substances that would interfere with the biuret reaction. The protein was precipitated with an equal volume of 10% w/v trichloracetic acid, the precipitate was dissolved in 30% w/v NaOH and the biuret colour developed by 5% w/v $CuSO_4$. The concentration of protein was determined by the use of a Lovibond comparator. The lower limit of sensitivity for this method was 30 mg./100 ml. protein.

In a few instances, where greater accuracy was required, a Gornall biuret reagent was used (Gornall, Bardawill and David, 1949) and the extinction read at 540 mμ in a Unicam SP 600 spectrophotometer. A calibration curve was prepared from normal human serum standardised by Kjeldahl nitrogen determination.

A microbiuret method

Levels of urinary protein below 30 mg./100 ml. were estimated by a microbiuret method, modified from that of Itzhaki and Gill (1964). This was a useful technique for estimating the total protein excretion in patients recovering from renal disease and in normal subjects. The Lowry method (Lowry et al., 1951), which would also be sufficiently sensitive,

Fig. 4. Protein standards estimated by the microbiuret method.

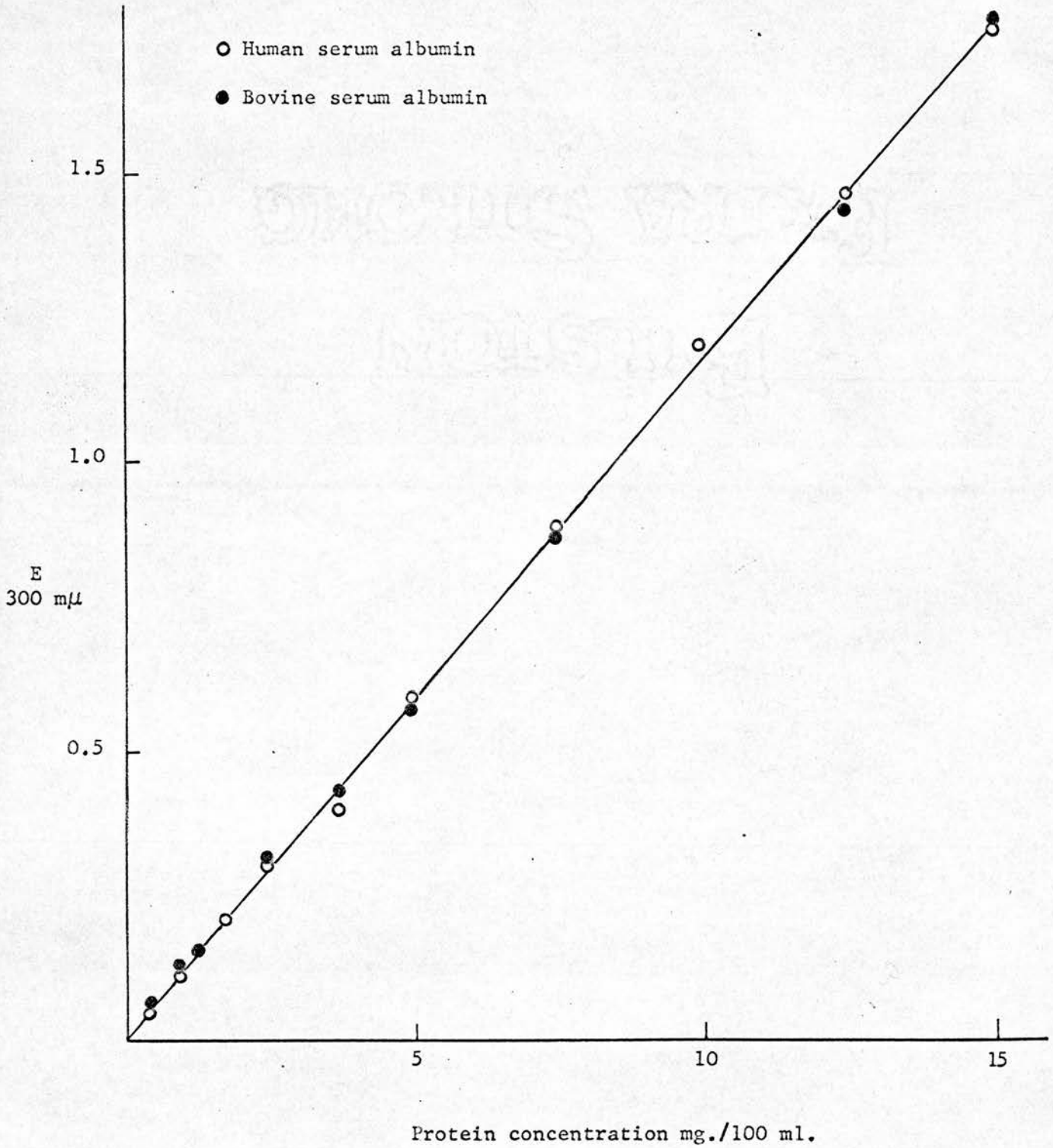


TABLE 1

Errors of the microbiuret method

(a) The protein concentrations of a 5 mg.% standard and a fresh normal urine were determined 10 times each and the mean reading, range of values, standard deviation (SD) and coefficient of variation (CV) are given.

Sample	Mean Reading E 280 mμ	Range	SD	CV %
Standard 5 mg.%	0.584 (5.05 mg.%)	0.640 - 0.545 (5.55-4.70 mg.%)	± 0.026	4.5
Fresh normal urine	0.315 (2.70 mg.%)	0.340 - 0.289 (2.95-2.50 mg.%)	± 0.016	5.1

(b) Protein concentrations of a 50 mg.% and a 30 mg.% standard, when approximately diluted, were estimated and the resulting errors are given.

Standard mg.%	Dilution factor	Reading E 280 mμ	Protein conc. mg.%	Error %
50	10	0.539	4.65	7
50	20	0.285	2.45	6
30	2	1.648	14.25	5
30	5	0.747	6.45	9

was not used because of the very different chromogenicity of different proteins (Saifer and Gerstenfeld, 1964).

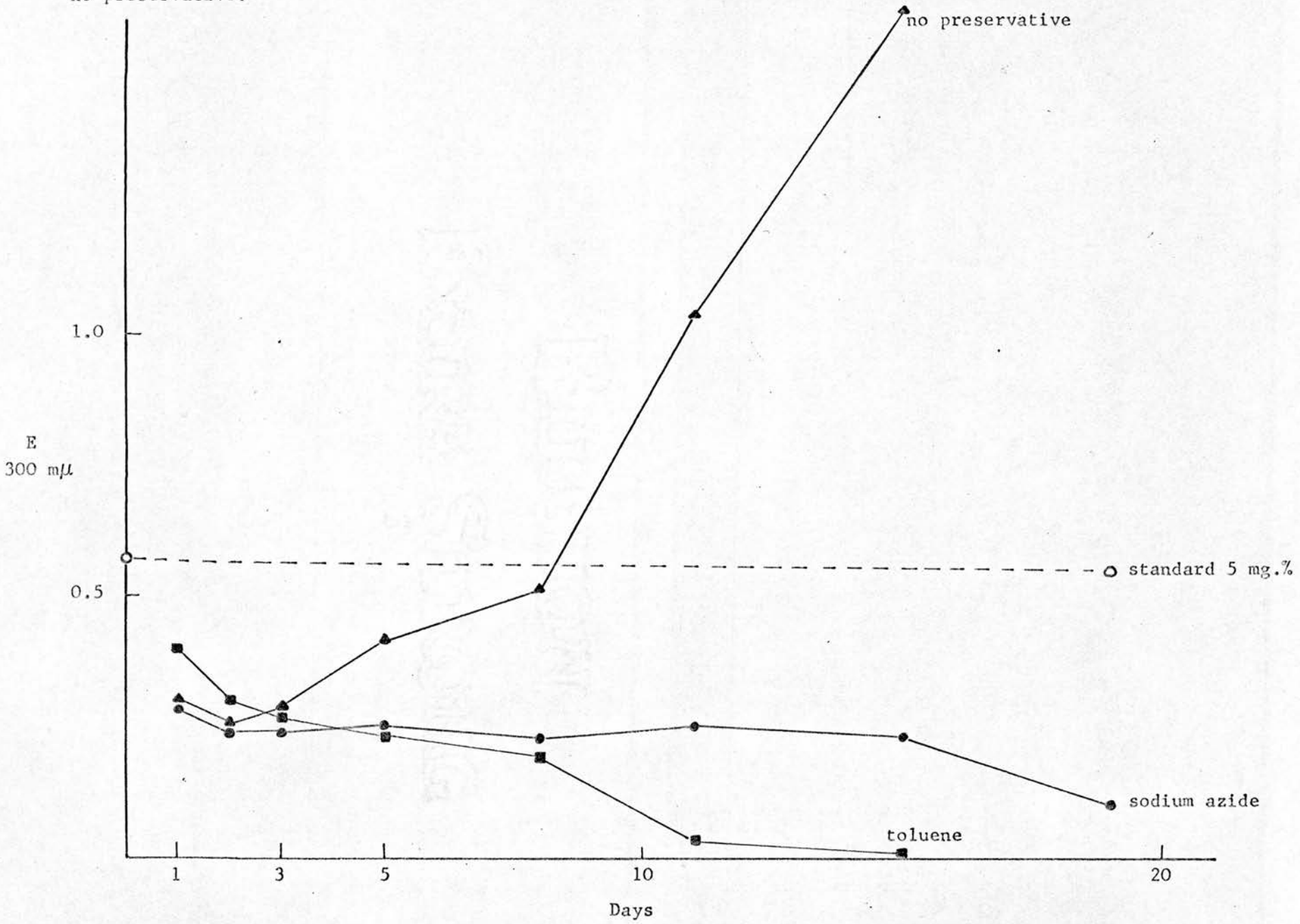
Urinary protein was first precipitated by trichloracetic acid, the precipitate was dissolved in an alkaline copper reagent and the extinction of the copper-protein complex was read at 300 mμ. The method was found to be simple, convenient and reproducible considering the small amounts of protein that were precipitated. It was not suitable for small volumes (less than 20 ml.) of urine.

Trichloracetic acid, 2 ml. of 30% w/v, was added to 10 ml. of fresh, filtered urine. The mixture was allowed to stand for 10 min. and was then centrifuged for 10 min. at 2000 rev./min. The supernatant was discarded and the tubes were inverted over absorbent tissue to drain for a few minutes. The precipitate was then washed with 2 ml. of 5% w/v trichloracetic acid, centrifuged, the supernatant discarded, and the tube drained as before. The precipitate was dissolved in 3 ml. of 0.07% w/v $CuSO_4$ in 10% w/v NaOH and the tube was centrifuged. The extinction was read in a Unicam SP 500 spectrophotometer at 300 mμ against the alkaline copper reagent as a blank.

Calibration curves were prepared from (1) a series of dilutions of human serum which was standardised by Kjeldahl nitrogen determination, (2) a series of standards prepared from crystalline bovine albumin. These calibrations were found to agree well and to be linear over the range 0 - 15 mg./100 ml. (Fig. 4). The reproducibility of the method was calculated from 10 estimations on each of 2 samples and found to be about 5%; the results are shown in Table 1. The error obtained when a sample of over 15 mg./100 ml. was diluted is also shown and was found to be of the same order of magnitude as the coefficient of variation of the method. As the coefficient of variation was high the assays on urine were always

Fig. 5. Stability of microbiuret estimations.

Microbiuret readings of samples of a normal urine stored at 4°C with added toluene, sodium azide or with no preservative.



performed in duplicate.

Bacterial contamination can contribute significantly to protein concentration, if the amounts of protein are small. The variation in protein concentration of a normal urine was therefore estimated over a period of days in the presence and absence of preservatives. All the samples were kept at 4°C and filtered before each estimation. The results are shown in Fig. 5. It can be seen that sodium azide is the only satisfactory preservative, and that the protein concentration in the presence of azide does not vary significantly up to 15 days.

Ultraviolet extinction

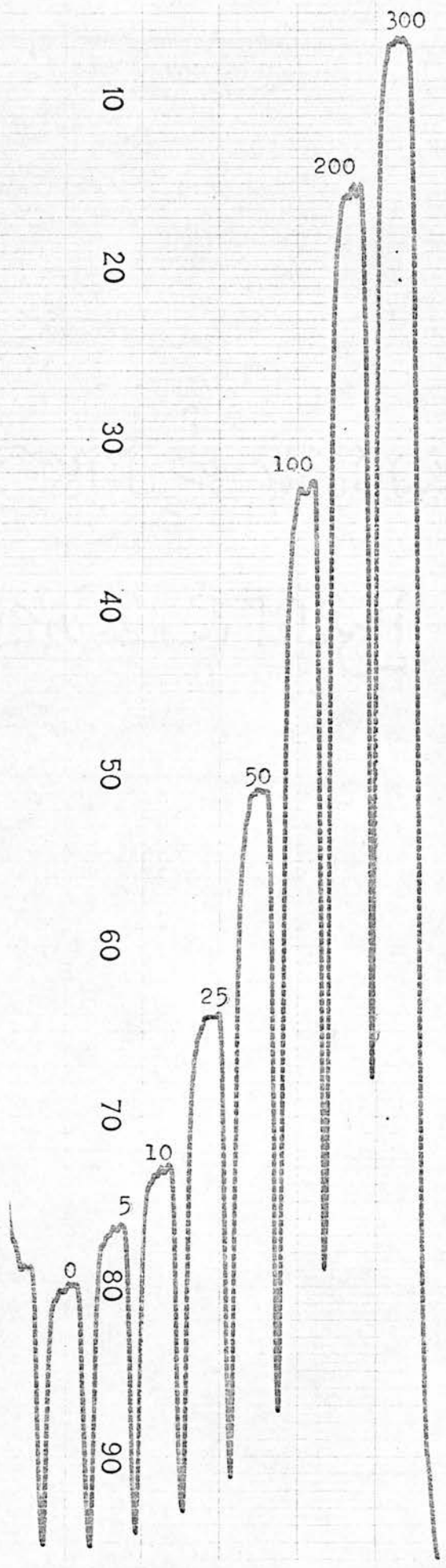
Of all the more sensitive ways of estimating protein, measurement of the extinction at 280 mμ (Warburg and Christian, 1941) is probably the simplest for a large number of samples. This method was used to assay the Sephadex column chromatographic effluents (2.3.3). The eluting buffer used in these experiments was 0.2M tris-saline pH 8.0, and protein concentrations in the effluents ranged from 0-2 mg./ml. Beer's Law is followed over this range and there is no significant interference by the tris buffer. Since absolute values of protein concentration were not required no calibration was carried out.

The extinction at 280 mμ depends on the aromatic amino acid (particularly tyrosine and tryptophan) concentration and therefore varies for individual proteins. However, the readings at 280 mμ were used to compare urine and serum proteins of the same molecular weight, and since a similar combination of proteins was being compared the error was probably minimal.

The Lowry method

Some of the column chromatographic effluents were assayed by Lowry's

Fig. 6. Protein standards estimated by the automated Folin-Ciocalteau method.



The peaks were recorded on an AutoAnalyzer and represent the colour developed by protein standards from diluted human serum, concentrations in mg./100 ml.

Folin-Ciocalteau method, adapted for an AutoAnalyzer (Mandl, 1961). This was useful if only small volumes of column effluents were available or if high lipid concentrations were causing turbidity (see 2.3.3). The tris-saline buffer was found to give a high background reading, but the method was still sufficiently sensitive over the range 0 - 300 mg./100 ml. protein. Fig. 6 shows a calibration curve obtained from a series of standards prepared from a normal serum pool. Absolute values of protein concentration were not required and the serum pool was estimated by a biuret reaction prior to dilution.

The Folin-Ciocalteau method preferentially measures certain amino acid sequences of proteins, but for the same reasons as given for ultra-violet absorption, this probably does not introduce any significant errors.

2.2.2. CONCENTRATION OF PROTEIN

Concentration of dilute solutions can be effected by freeze drying, osmotic ultrafiltration or pressure ultrafiltration. Irreversible denaturation can occur on freeze drying and pressure ultrafiltration is inconveniently slow. Osmotic ultrafiltration, however, has neither of these disadvantages. In most cases concentration of protein for selectivity studies was carried out by this method. In a few instances pressure ultrafiltration was also used, in order to confirm that the material used for osmotic ultrafiltration had no effect on the protein being concentrated.

Osmotic ultrafiltration

Protein solutions were concentrated by dialysis against polyethylene glycol, Carbowax 20M, (PEG). This method was rapid and convenient. Depending on the protein concentration of the untreated urine a suitable aliquot was either filtered or centrifuged at 2000 rev./min. for 10 min. The urine was placed in Visking dialysis tubing (18/32 or 36/32), dialysed against running tap water overnight, and then placed in a solution of PEG. Different sizes of Visking dialysis tubing have been noted to have different permeabilities, but this has no significant effect where molecular weights of over 40,000 are concerned (Berggard, 1961a, 1961b). The dialysis tubing was always boiled and washed in distilled water prior to use. The rate of concentration depended on the strength of the PEG solution, but it was possible to reduce the volume by 50 ml. in 2 - 3 hours. The tubing was then washed well to remove the PEG, and the knot at one end cut. A small quantity of appropriate buffer was added to wash all the contained protein down to the bottom of the tubing, the empty tubing was cut off and the part containing the concentrated protein solution was inverted over a small test tube and gently centrifuged.

TABLE 2

Experiments with polyethylene glycol

(a) Amount of PEG counter-dialysing

Vol. of dist. H_2O conc. by PEG (ml.)	Dialysis tubing used	Vol. of final solution (ml.)	Conc. PEG in final solution (g./100 ml.)	Total PEG counter-dialysed (mg.)	Amount of PEG left after dialysis and centrifugation (mg.)
1000	36/32	10.0	0.35	35	17
1000	36/32	6.0	0.70	42	
1000	36/32	4.5	1.3	59	25
750	18/32	4.0	0.40	16	
150 saline	18/32	2.0	0.65	13	8

(b) Effect of PEG on estimation of protein

PEG mg.%	E 280 mμ	Reading of 50 mg.% protein standard containing PEG, by Folin-Ciocalteau method
500	1.645	150
250	0.840	
100	0.335	83
50	0.165	
25	0.085	76
10	0.038	60
5		56
2.5		53
1.75		50
0.875		50

Loss of protein through the dialysis tubing was tested for by concentrating a measured volume of urine down to 1 ml. and then diluting the concentrate back to the original volume. Protein in both the treated and untreated urine was estimated by the Gornall biuret reaction (2.2.1). The mean loss of protein from 6 samples was 1.6%, a range of volumes between 150 and 20 ml. being concentrated.

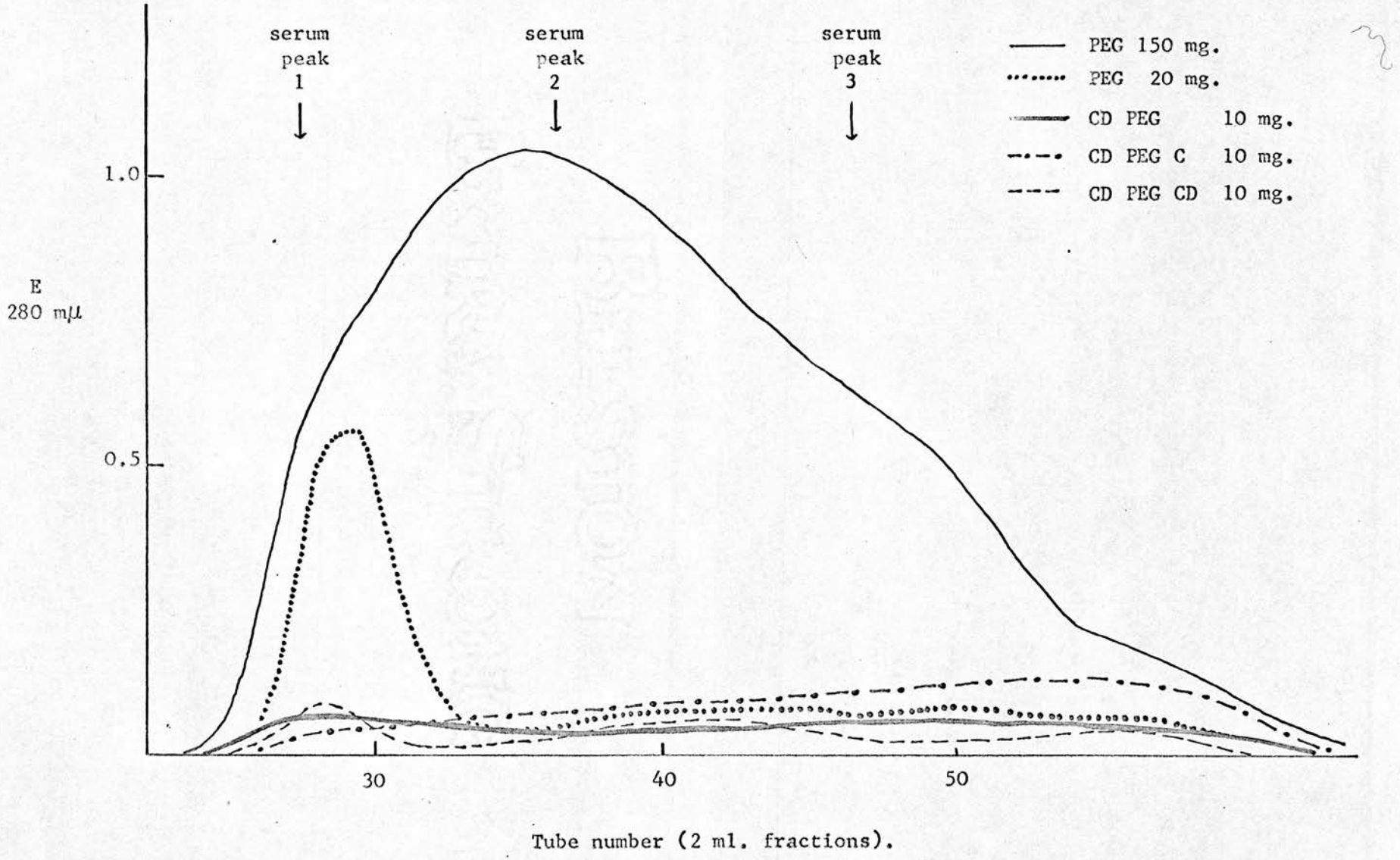
PEG was found to interfere with protein estimation by giving extinction readings at 280 mμ and by causing precipitation in the Folin-Ciocalteau reaction. Moreover, on column chromatography using Sephadex G 200 it was found to be eluted over the protein range. Since it has been reported to counter-dialyse (Howe, Groom and Carter, 1964), protein solutions concentrated by PEG will contain some PEG, and the effluents from Sephadex column chromatography might therefore give spuriously high protein readings. Some experiments were carried out to ascertain the amount of counter-dialysis, and the effect on column chromatography.

Distilled water was "concentrated" in exactly the same way as protein and the extinction of the final solution was measured in a Unicam SP 500 spectrophotometer at 280 mμ. The amount of PEG which had counter-dialysed was estimated from a calibration curve, obtained from measuring the extinction at 280 mμ of a series of solutions (0.5 - 0.05 g./100 ml.) of PEG. A control was run by placing distilled water in a dialysis tubing during the same period and then measuring the extinction of the water at 280 mμ. Solutions of PEG were also estimated by the Folin-Ciocalteau reaction to determine the limit of detection. PEG and counter-dialysed PEG were applied to a Sephadex G 200 column (2.3.3) and the effluents were read at 280 mμ.

The results are summarised in Table 2 and Fig. 7. It can be seen that small amounts of PEG, of the order of 30 - 40 mg./litre can enter the

Fig. 7. Gel filtration of PEG and counter-dialysed PEG.

Some of the counter-dialysed PEG (CD PEG) was centrifuged (CD PEG C) or centrifuged and dialysed (CD PEG CD) before application to the Sephadex G 200 column. The position of the three serum protein peaks is also shown.



dialysis tubing, but that about half can be eliminated by prolonged dialysis and centrifugation. PEG affects readings at 280 mμ and on the Folin-Ciocalteau reaction at concentrations of 2.5 mg.% and over. On Sephadex, counter-dialysed PEG, whether or not it was first dialysed and centrifuged, was found to elute over the same high molecular weight protein range as untreated PEG. These strange elution patterns of PEG on Sephadex G 200 have also been described by Ryle (1965). It can only be concluded that there is dynamic equilibrium between the small and large molecular weight forms, or alternatively that Sephadex has some aggregating effect on the PEG.

If, from the results in Table 2 and Fig. 7, the concentration of PEG in a protein concentrate was about 4 mg./ml. and 1 ml. was applied to a Sephadex column, the PEG concentration in the effluents would be diluted about 70 times and would average perhaps 3 mg.%. This amount would not appreciably affect readings either at 280 mμ or by the Folin-Ciocalteau method.

The effect of PEG on immunological reactions was tested. A serum was diluted with saline and concentrated by PEG to the original volume. The treated and untreated sera were then compared by immunoelectrophoresis against antihuman serum (2.3.2) and by immunodiffusion against several specific antisera (2.3.1). A urine sample with a urinary protein concentration of 280 mg.% was treated in the same way. No differences could be detected between the treated and untreated samples, a finding which agrees with that of Howe et al. (1964).

Pressure ultrafiltration

Because of the counter-dialysis problem using PEG, in a few cases the urinary protein was also concentrated by pressure ultrafiltration, using 2

or 3 LKB ultrafilters in parallel. The method was found to be slow, and less convenient than concentration by PEG. A maximal rate of about 12 ml. per hour, per ultrafilter, could be obtained.

The urine was either filtered or centrifuged at 2000 rev./min., dialysed against running tap water overnight and then transferred to a suitable container. Dialysis tubing, which was supported by a plastic frame, was placed in the urine and negative pressure was applied to the inside of the tubing. The apparatus then began filtering, the ultrafiltrate was removed by the suction and the volume of urine was gradually reduced. The method was not practical when small volumes of urine were available, since reasonable rates were only obtained when a maximal surface area for filtration was operating.

Loss of protein was tested for by reversing the process; urine was placed inside the tubing and negative pressure was applied outside. The ultrafiltrate was retained and the protein concentration compared to that of the final concentrate. Loss of protein was small, less than 1% of the original protein was recovered in the ultrafiltrate. Overall recovery, however, was low, the final amount of protein in the concentrate was only 71% of that in the untreated urine. Low recoveries were also obtained by McGarry et al., (1955), who concluded that this was due to firm adherence of protein to the interstices of the filter membranes.

2.3. SELECTIVITY STUDIES USING ENDOGENOUS MACROMOLECULES

An immunodiffusion technique was first used by Blainey et al. (1960) to determine selectivity of proteinuria and this was the principal method used in this study. However, it is only practicable to estimate the clearance of a few proteins by this method and the error of the individual estimations is high (Soothill, 1962). Moreover the method assumes a molecular weight for each protein, whereas in theory degraded fragments of the parent protein molecules could also react antigenically and give spuriously high results.

With the introduction of gel filtration it has become possible to estimate molecular weight distribution by column chromatography, and by applying this technique to serum and urine, selectivity values based entirely on molecular weight can be determined. However, unlike immunological methods, gel filtration cannot discriminate specifically between proteins derived from the serum, and proteins derived from other sources.

Both methods have inherent limitations but are complementary to one another. Since the principles on which they are based are quite different, if the results obtained by immunodiffusion correlate weil with those obtained by gel filtration it may be concluded that selectivity of proteinuria is an estimate of renal permeability in terms of molecular weight. The reproducibility of the index of selectivity and possible sources of error were fully assessed for each method. Immunoelectrophoresis was used to study selectivity on a qualitative basis and clearances of some enzymes were also determined.

2.3.1. IMMUNODIFFUSION

Immunological methods were used to identify and quantitate proteins of known molecular weight in serum and urine by allowing precipitation reactions to take place with specific antisera in agar gel. The theory and applications of diffusion-in-gel methods for immunological analysis are reviewed by Ouchterlony (1962).

There are many precise methods of quantitating antibody-antigen precipitin complexes, but for the purposes of estimating selectivity the simple visual principle of Gell (1957) was chosen. This was adapted by Soothill (1962) for serum and urine assays and used by Blainey et al. (1960) to estimate renal permeability to different proteins in patients with the nephrotic syndrome, when the concept of selectivity was first introduced.

The method involves a double diffusion of antibody and corresponding antigen in agar gel. If a specific antiserum is used, a single precipitation line forms, the amount of precipitation being a function of both antibody and antigen concentration. By using a fixed range of antibody concentrations against different antigen concentrations and comparing the precipitin lines, a direct quantitative comparison of the amounts of antigen can be made. In this way urine:serum ratios of individual proteins of known molecular weight and hence selectivity can be estimated.

Details of the method

The method is essentially that of Soothill (1962), but is described here in detail with the modifications that were introduced.

A solution of 1% w/v agar in 0.2M phosphate-saline buffer pH 7.0 was prepared by heating over a boiling water bath. The agar was filtered and poured into flat-bottomed Petri dishes, diameter 6.5 cm. to a depth of 2.5mm. The poured Petri dishes could be kept at room temperature for about 10 days

before drying out began. The buffer contained 0.01% w/v sodium azide to prevent bacterial growth.

A pattern of six large holes each surrounded by six small holes, was cut in the agar by a special template (see Fig. 8). Dilutions of antiserum (Behringwerke) were placed in the small holes, the concentration of antiserum increasing from a - f. Doubling dilutions of serum (1 - 4) and urine (5,6) were placed in the larger holes. The dilutions were made up in the 0.2M phosphate-saline buffer by a drop method using a Pasteur pipette with an even tip. Usually one drop of the appropriate dilution was added to each hole, but occasionally two or four drops were added when the urine antigen concentration was very low.

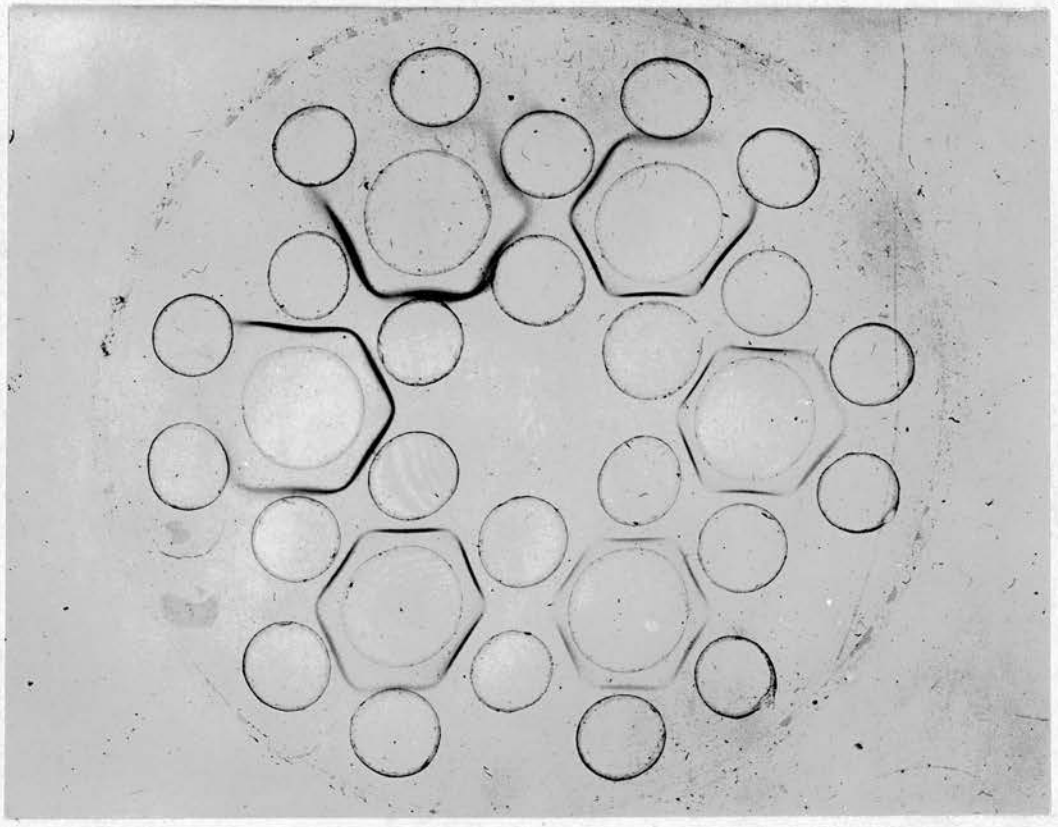
Initially, the most suitable range of dilutions for each antiserum and the corresponding range of dilutions for serum and urine were found by a systematic trial. The antiserum and serum dilutions were not usually varied, except when the potency of the antiserum altered or when the serum had an unusually high or low antigen concentration. The urine dilutions depended on the amount of protein in the urine and a series of dilutions was therefore worked out for different Albustix (2.2.1) concentrations.

Reading of the results

After allowing precipitation to take place for 24 - 48 hours, the antigen-antibody precipitation lines were seen as hexagons surrounding each large hole. The plates were examined by oblique illumination against a black background on a special viewing box. It was found that precipitin hexagons for the smaller proteins (albumin, transferrin, γ-globulin) were fully formed after 24 hours, but for the larger proteins (α_2-macroglobulin, β-lipoprotein) precipitation was not complete until 48 hours. If necessary, 2% acetic acid was added to enhance the precipitin lines.

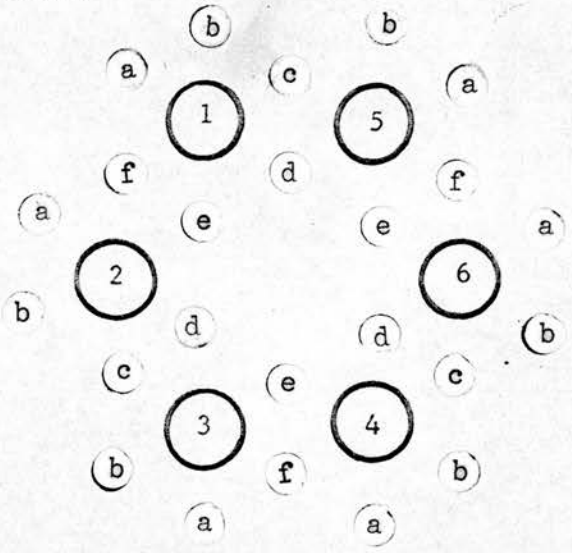
Fig. 8. Estimation of urine:serum ratios by immunodiffusion.

A dried stained Ouchterlony plate



Urine:serum ratios were estimated by comparing the thickness, intensity and position of the precipitin lines. The preparation was stained by Ponceau S.

Identification chart



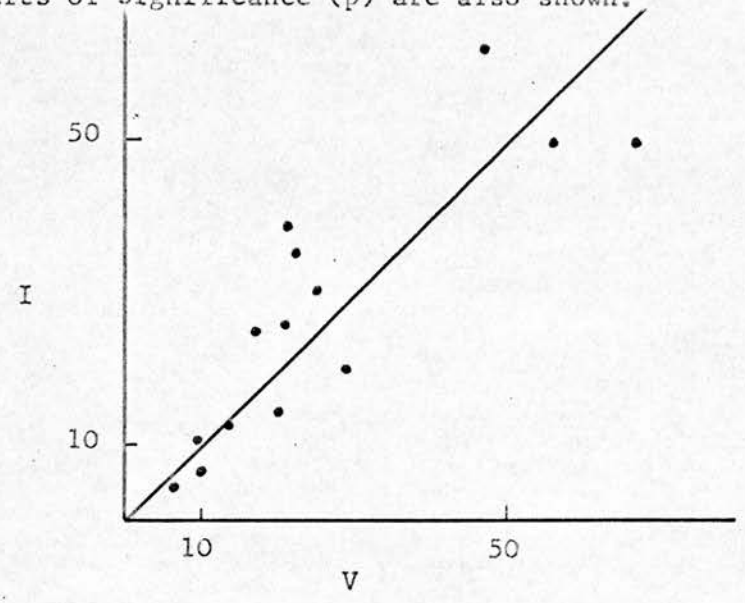
Serum dilutions were placed in holes 1 - 4 and urine dilutions in holes 5 and 6. Antisera dilutions were placed in holes a - f, the concentration in a being the weakest.

A dried stained plate is shown in Fig. 8. The two precipitation curves of fixed antibody against increasing antigen concentration, and fixed antigen against increasing antibody concentration are superimposed. The result is that when the precipitation lines are studied it can be seen that the strongest serum dilution (1) tends to have optimum precipitation with the strongest antiserum (f) and the weakest serum dilution (4) with the weakest antisera (a, b). The distance of the precipitation line from the antigen hole is also related to both antibody and antigen concentration. By comparing the thickness, intensity and position of the precipitation lines in relation to antibody dilution it is possible to match the serum and urine hexagons. In this case the urine dilution 5 was weaker than serum dilution 2, and stronger than serum dilution 3. Similarly the urine dilution 6 was weaker than serum dilution 3 and stronger than serum dilution 4. The serum dilutions were (1) 1/128, (2) 1/256, (3) 1/512, (4) 1/1024 and the urine dilutions were (5) 1/16, (6) 1/32. Three arithmetic interpolations were made when the urine hexagons did not exactly match the serum hexagons. For example the urine dilution of 1/16 equivalent to the serum dilution of between 1/256 and 1/512 would read as 1/320, 1/384, or 1/448 so that the urine:serum ratio would be 1:20, 1:24 or 1:28. In this case the urine concentration was read as mid-way between the serum hexagons on the plate, the antisera was γ-globulin and the urine:serum ratio for γ-globulin therefore read as 1:24.

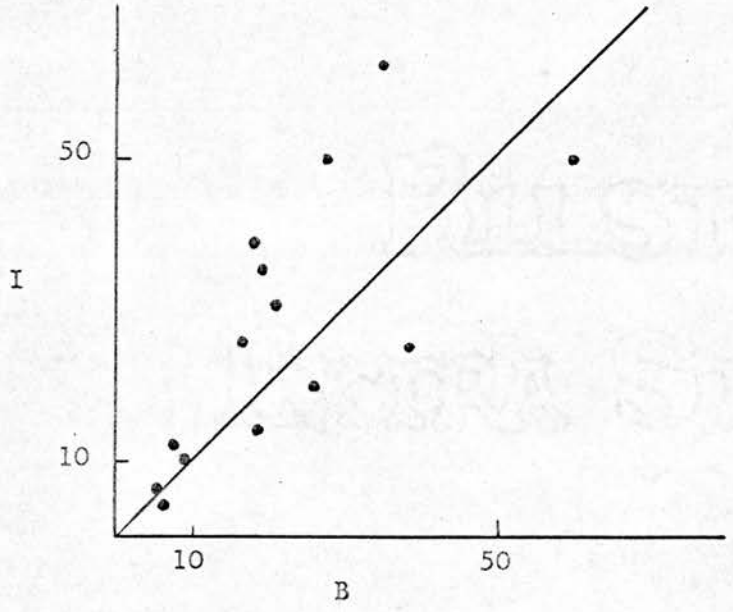
The specificity of each batch of antisera was checked by immuno-electrophoresis (2.3.2) and spurs or reactions of partial identity were never seen. In the great majority of cases the Albustix reading (2.2.1) reflected the urinary protein concentration sufficiently accurately.

Fig. 9. Correlation of different methods of determination of the concentration factor.

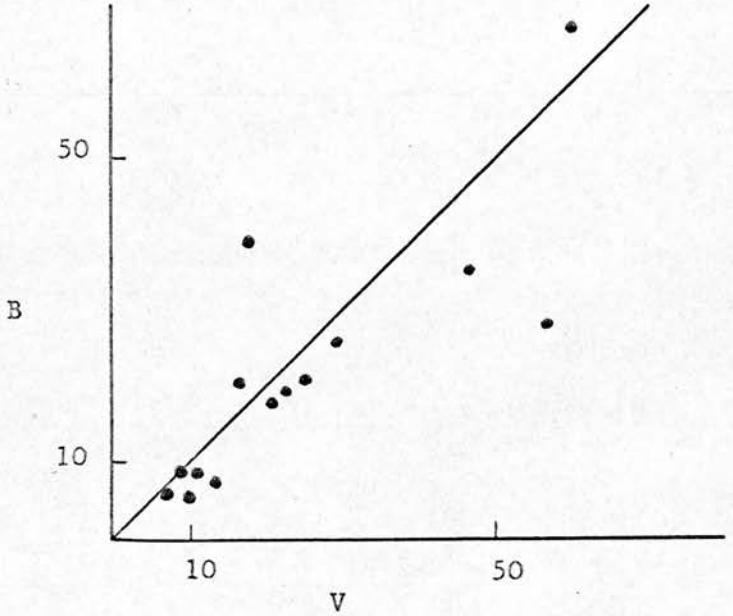
The concentration factor was determined immunologically (I), by biuret determinations (B) and by volume measurements (V). The correlation coefficient (r) and its limits of significance (p) are also shown.



$r = +0.845$, $p < 0.001$



$r = +0.713$, $p < 0.005$



$r = +0.826$, $p < 0.001$

Occasionally, however, the urine dilutions were too weak or too strong and the determination was repeated using more appropriate dilutions.

Calculation of urine:serum ratios

Urine:serum ratios were estimated in this way for five individual proteins: albumin, transferrin, γ-globulin, α_2-macroglobulin and β-lipoprotein. Table 3 gives details of all the urine and serum dilutions used and the readings made in a typical experiment. The usual antiserum dilutions are also given. The first three proteins were generally detected in untreated urine, but α_2-macroglobulin and β-lipoprotein were not usually seen unless the urine was first concentrated (2.2.2), and even on concentration β-lipoprotein was not always detected, particularly if the urinary protein concentration was low.

A volume of urine containing approx. 150 mg. protein was concentrated to approx. 1 ml. and the concentrate was then added to the plates instead of urine. In order to calculate urine:serum ratios for α_2-macroglobulin and β-lipoprotein the degree of concentration of the urine was estimated immunologically. Two dilutions of urine concentrate and four dilutions of untreated urine were set up against anti-human serum. The plate was read in exactly the same way as the others by matching the hexagons. For example, if a concentrate is diluted by 1000 and the hexagon formed by 1 drop of this dilution is equivalent to a hexagon formed by 1 drop of a urine dilution of $^1/_{28}$, the concentration factor is then 36. Figures for the concentration factor obtained by this immunological method agreed reasonably well with those by volume measurements and by the determination of total protein by the Gornall biuret method (2.2.1). The correlation is shown in Fig. 9. The concentration factor was used to convert urine concentrate:serum ratios to urine :serum ratios.

TABLE 3

Examples of dilutions used and readings made in the immunodiffusion method of determination of selectivity of proteinuria

(a) Determination of selectivity

Plate No.	Antisera to	Serum Dilutions	Urine Dilutions	Hexagons Matching	Urine / Serum	% clearance	log (10x% clearance)	log (mol.wt. of protein)
1	Albumin	$\frac{1}{256}$ $\frac{1}{512}$ $\frac{1}{1024}$ $\frac{1}{2048}$	$\frac{1}{32}$ $\frac{1}{64}$	$\frac{U}{32}=\frac{S}{640}$, $\frac{U}{64}=\frac{S}{1280}$	$\frac{1}{20}$	100	3.00	4.84
2	Transferrin	$\frac{1}{32}$ $\frac{1}{64}$ $\frac{1}{128}$ $\frac{1}{256}$	$\frac{1}{2}$ $\frac{1}{4}$	$\frac{U}{2}=\frac{S}{48}$, $\frac{U}{4}=\frac{S}{96}$	$\frac{1}{24}$	83	2.92	4.95
3	γ-Globulin	$\frac{1}{128}$ $\frac{1}{256}$ $\frac{1}{512}$ $\frac{1}{1024}$	$\frac{1}{2}$ $\frac{1}{4}$	$\frac{U}{2}=\frac{S}{192}$, $\frac{U}{4}=\frac{S}{384}$	$\frac{1}{96}$	21	2.32	5.18
4	α_2-Macro-globulin	$\frac{1}{32}$ $\frac{1}{64}$ $\frac{1}{128}$ $\frac{1}{256}$	$\frac{C}{4}$ C	$\frac{C}{4}=\frac{S}{128}$, $C=\frac{S}{32}$	$\frac{1}{1440}$	1.4	1.15	5.92
5	β-Lipoprotein	$\frac{1}{16}$ $\frac{1}{32}$ $\frac{1}{64}$ $\frac{1}{128}$	C 4C	$C=\frac{S}{160}$, $4C=\frac{S}{40}$	$\frac{1}{7200}$	0.27	0.43	6.40
6	Human serum	Dilution of C $=\frac{C}{2500}$	$\frac{1}{16}$ $\frac{1}{32}$ $\frac{1}{64}$ $\frac{1}{128}$	$\frac{C}{2500}=\frac{U}{56}$ $\therefore C = 45U$				

S = serum, U = urine, C = urine concentrate.
In this sample the urine Albustix reading was 300 mg.% and the proteinuria was of intermediate selectivity with -k = 1.68.

(b) Antisera dilutions

	a	b	c	d	e	f
Antisera to all proteins except γ-Globulin	$\frac{1}{40}$	$\frac{1}{20}$	$\frac{3}{40}$	$\frac{1}{10}$	$\frac{1}{8}$	$\frac{3}{20}$
Anti γ-Globulin serum	$\frac{3}{40}$	$\frac{1}{10}$	$\frac{1}{8}$	$\frac{3}{20}$	$\frac{7}{40}$	$\frac{1}{5}$

A direct check on the concentration procedure was possible when the urine sample contained a high concentration of α_2-macroglobulin. The urine:serum ratio for this protein could then be estimated using both untreated and concentrated urine and the values compared. The clearance of α_2-macroglobulin calculated from the concentrated urine tended to be significantly lower than the value calculated from the untreated urine. However, the mean difference from twenty pairs of observations was 12%, which was within the reproducibility of the method. The coefficient of variation calculated from the twenty pairs of observations was 18%, a figure which is very close to the coefficient of variation of the estimation given in Table 6.

In urine samples where no protein was detectable by Albustix, a litre was concentrated until a positive Albustix reading was obtained. The volume was measured to obtain the first degree of concentration. This first concentrate was then treated as urine and a second concentrate prepared. Urine:serum ratios were determined as described above, using the first concentrate, as urine, to estimate albumin, transferrin and γ-globulin and the second concentrate to estimate α_2-macroglobulin and β-lipoprotein. The second concentration factor was determined in the normal way. The urine concentrate:serum ratios were then converted to urine:serum ratios by the first and second concentration factors.

Calculation of selectivity

The clearances of all the proteins were expressed as a percentage of the albumin urine:serum ratio (Table 3). In this way the relative clearances obtained from serum and urine samples of different protein concentrations could be compared. When the relative clearances of the individual proteins are plotted against molecular weight on a log-log scale an

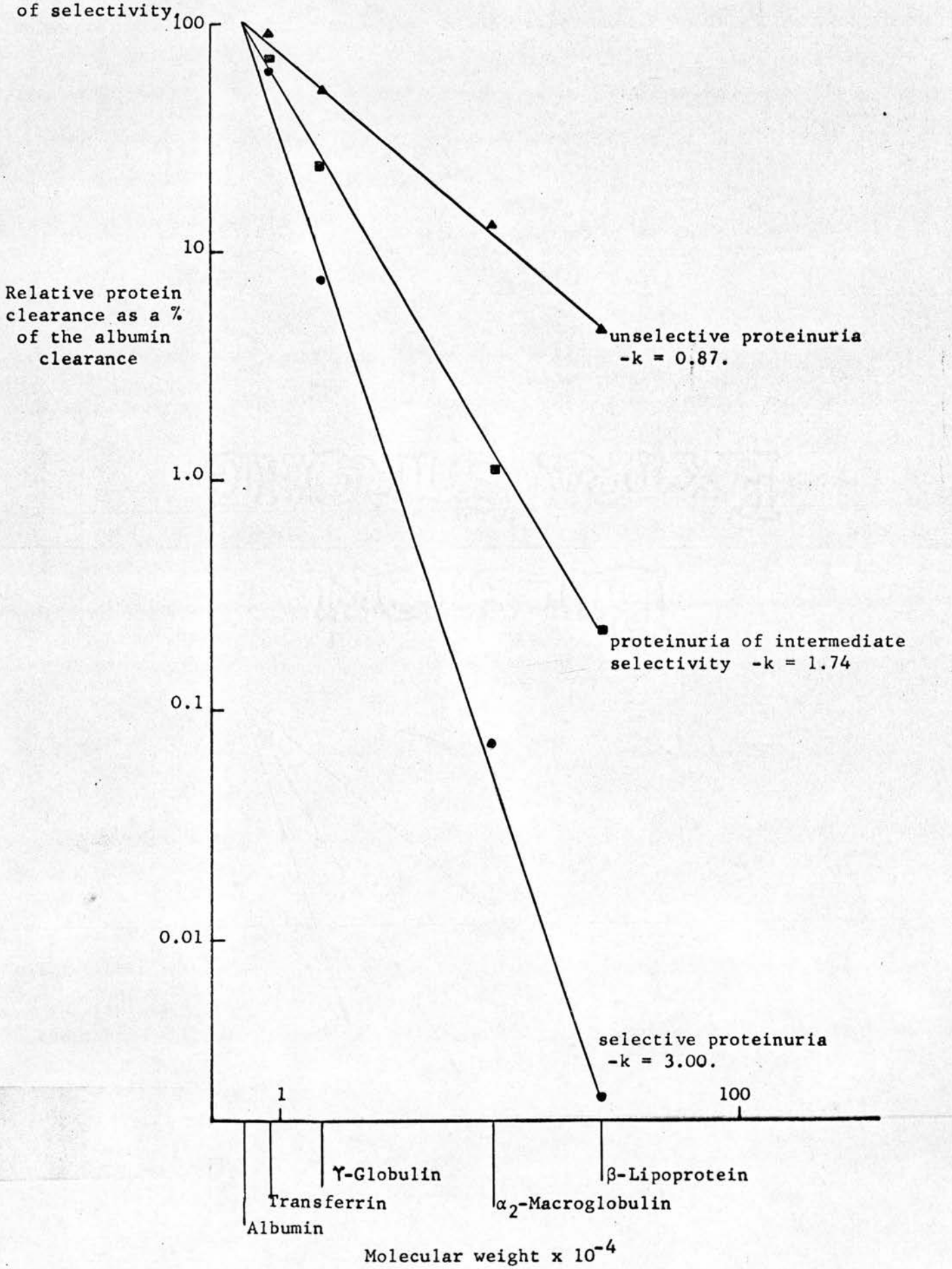
TABLE 4

Molecular weights of the proteins used in the determination of selectivity of proteinuria by immunodiffusion and immunoelectrophoresis

Protein	Molecular weight	Reference
Orosomucoid	44 000	Smith et al. (1950)
α_1-Glycoprotein	54 000	Phelps and Putnam (1960)
Prealbumin	61 000	Winzler (1960)
Albumin	69 000	Phelps and Putnam (1960)
Transferrin	90 000	Koechlin (1952)
γ-Globulin	150 000	Isliker (1957)
Haptoglobin	89 000 or 100 000 and dimers	Shim and Bearn (1964)
Ceruloplasmin	150 000	Winzler (1960)
γ_{1A}-Globulin	160 000	Fahey (1962)
α_1-Lipoprotein	200 000	Schultze et al. (1957)
Fibrinogen	330 000	Scherega and Laskowski (1957)
α_2-Macroglobulin	840 000	Schultze (1957)
γ_{1M}-Globulin	1000 000	Fahey (1962)
β-Lipoprotein	2500 000	Shore (1957)

Fig. 10. Determination of selectivity of proteinuria by immunodiffusion.

Relative protein clearances determined by immunodiffusion plotted against molecular weight on a log-log scale. The calculated slope of the line = k, and -k = the index of selectivity.



approximately linear relationship is found. The molecular weights of the proteins are given in Table 4 and Fig. 10 shows some typical log-log plots. The slope of the line is an index of the selectivity of the proteinuria. A selective proteinuria contains a small proportion of high molecular weight protein and the slope of the line (k) is very steep. Conversely an unselective proteinuria contains a large proportion of high molecular weight protein and the slope of the line is much flatter. The index of selectivity, k, (the slope of the line) was calculated by estimating the regression line by the method of least squares. For ease of calculation the relative clearances of the individual proteins were multiplied by 10 or 100 before converting into logarithms, so that the albumin clearance of 100% was 3.00 or 4.00 and the clearances of the other proteins in logarithms were also changed accordingly (Table 3).

Errors of the method

The errors involved in estimating urine:serum ratios in this way are high (Soothill, 1962), but the error of the determination of selectivity has not been measured. The accuracy of the method was therefore investigated in several ways, in an attempt to discover the factors responsible for the large error and to estimate the overall error of selectivity measurements.

The drop dilution method is quick, convenient and requires a very small sample, but it is not very accurate. The error was assessed by weighing a counted number of drops. The coefficient of variation was found to be 7.8%.

Since in some cases 2 or 4 drops of urine or urine concentrate were added to the antigen holes a plate was set up to compare the effect of adding a larger volume of antigen. Serum dilutions of $\frac{1}{512}$, $\frac{2}{2048}$ and $\frac{4}{4096}$ were set up against anti-albumin. No detectable difference could be

TABLE 5

A theoretical calculation of the errors involved in the determination of selectivity of proteinuria

(a) Details of individual protein clearances

Typical values of clearances of individual proteins - albumin (A), transferrin(S), γ-globulin (γ), α_2-macroglobulin (α_2), and β-lipoprotein (β) - were used to calculate a value for the index of selectivity, -k = 1.53. S = serum, U = urine, C = urine concentrate, CF = concentration factor.

	A	S	γ	α_2	β	CF
Reading	$\frac{U}{256} = \frac{S}{1024}$	$\frac{U}{32} = \frac{S}{160}$	$\frac{U}{32} = \frac{S}{512}$	$4U = \frac{S}{64}$	$C = \frac{S}{32}$	$C = 25U$
U/S	$\frac{1}{4}$	$\frac{1}{5}$	$\frac{1}{16}$	$\frac{1}{256}$	$\frac{1}{800}$	
% clearance	100	80	25	1.6	0.50	

(b) Error introduced when urine:serum ratios were mis-read.

The error in -k was calculated when clearances of individual proteins were mis-read by one or two steps.

Clearance changed 1 step	-k	% Error	Clearance changed 2 steps	-k	% Error
$A = \frac{1}{5}$	1.50	2.3	$A = \frac{1}{6}$	1.47	4.1
$A = \frac{1}{3.5}$	1.55	1.4	$A = \frac{1}{3}$	1.58	2.9
$S = \frac{1}{6}$	1.51	1.5	$S = \frac{1}{3.5}$	1.58	2.9
$S = \frac{1}{4}$	1.56	1.8	$S = \frac{1}{7}$	1.49	2.6
$\gamma = \frac{1}{20}$	1.52	1.0	$\gamma = \frac{1}{12}$	1.55	1.2
$\gamma = \frac{1}{14}$	1.54	0.6	$\gamma = \frac{1}{24}$	1.50	1.8
$\alpha_2 = \frac{1}{320}$	1.55	1.5	$\alpha_2 = \frac{1}{384}$	1.58	2.8
$\alpha_2 = \frac{1}{224}$	1.52	1.1	$\alpha_2 = \frac{1}{192}$	1.50	2.2
$\beta = \frac{1}{1000}$	1.58	3.4	$\beta = \frac{1}{1200}$	1.63	4.1
$\beta = \frac{1}{700}$	1.50	2.0	$\beta = \frac{1}{600}$	1.47	6.1
$C = 20U$	1.48	3.4	$C = 15U$	1.42	7.4
$C = 30U$	1.57	2.7	$C = 35U$	1.61	5.1
$A = \frac{1}{5}$ $\beta = \frac{1}{700}$	1.47	4.0	$A = \frac{1}{6}$ $\beta = \frac{1}{600}$	1.41	8.2
$A = \frac{1}{3.5}$ $\beta = \frac{1}{1000}$	1.60	4.7	$A = \frac{1}{3}$ $\beta = \frac{1}{1200}$	1.66	8.6

TABLE 6

Experimental errors in the determination of selectivity of proteinuria by the immunodiffusion method

Analyses were carried out either in duplicate or repetitively in order to calculate standard deviations (SD) and coefficients of variation (CV) for the estimations of relative clearance and selectivity. S = serum, U = urine.

Analysis	No. of readings	Entity	Mean	Range	SD	CV %	CV in logs %
Estimations in duplicate	32 pairs	Albumin S:U	10.5	1.75 - 80	± 2.23	21	
	30 pairs	Relative transferrin clearance	87	29 - 200	± 16.8	19	2.6
	24 pairs	Relative γ-globulin clearance	20	3.1 - 50	± 3.46	17	2.6
	13 pairs	Relative α_2-macroglobulin clearance	2.1	5.2 - 0.27	± 0.356	17	6.1
	12 pairs	Relative β-lipoprotein clearance	0.76	4.0 - 0.07	± 0.109	14	8.0
	10 pairs	Selectivity	1.64	1.18-2.49	± 0.061	3.7	
Several estimations on one serum and one urine (with a separate urine concentrate for each estimation)	10	Albumin S:U	12	10 - 16	± 1.9	16	
	10	Relative transferrin clearance	91	83 - 100	± 7.6	8.4	1.4
	10	Relative γ-globulin clearance	22	18 - 25	± 2.5	11	1.7
	10	Relative α_2-macroglobulin clearance	3.6	3.2 - 3.9	± 0.27	7.3	2.6
	10	Relative β-lipoprotein clearance	0.46	0.36-0.56	± 0.052	11	7.6
	10	Selectivity	1.47	1.43-1.52	± 0.028	1.9	
Several estimations on one urine and the corresponding concentrate	10	Concentration factor	56	48 - 64	± 5.0	9.7	
	5	Concentration factor	22	20 - 26	± 2.4	11	
Comparison of random and 24 hour urine collections	5 pairs	Selectivity	1.80	1.60-2.11	± 0.095	5.3	

seen in the hexagons. Adding more antigen at 16 hours, however, produced a faint doubling of the precipitin lines. If an antigen was not visible, the plate was therefore repeated using a higher concentration, rather than adding more antigen to the first plate.

The limit of the sensitivity of the immunodiffusion method rests at the differentiation between arithmetic steps, e.g. $^{1}/40$ and $^{1}/48$; it was found that further arithmetic interpolations as made by Joachim et al. (1965) were not feasible. Since visual matching of the hexagons is not precise the result could easily be "one step" out, i.e. the urine hexagon could be read as $^{1}/40$ instead of $^{1}/48$. A theoretical calculation of the errors involved was made. The results are shown in Table 5. It can be seen that reading any one plate "one step" out results in a maximum error in the index of selectivity of 3.4% and two steps out gives a maximum error of 7.4%. Various combinations of different readings result in errors of the same order of magnitude.

The reproducibility of the urine:serum ratio was calculated from a series of estimations performed in duplicate for all five proteins. The reproducibility of the selectivity value was also calculated in the same way from pairs of duplicate values. In addition, a series of ten estimations on the same serum and urine samples was determined to give the reproducibility of both the urine:serum ratios and selectivity values. A separate urine concentration was carried out for each of the ten determinations. Ten estimations on one urine and its corresponding urine concentrate, and five estimations on another, also gave the reproducibility of estimation of the concentration factor. The results are summarised in Table 6. Selectivity values from random and 24 hour urine collections were also compared. It can be seen that the coefficients of variation for the individual urine:serum

TABLE 7

The linear relationship between relative clearance and molecular weight of protein on a log-log plot by immunodiffusion

The correlation coefficient (r), its limits of significance (p) and the standard error of estimate of the relative clearances of individual proteins (SE) were calculated in 30 subjects, with varying degrees of proteinuria, as indicated by the albumin serum:urine ratio (Alb S:U). The index of selectivity (-k) and the relative clearances of the individual proteins are also given: transferrin (S), Υ-globulin (Υ), α_2-macroglobulin (α_2) and β-lipoprotein (β).

Subject	-k	Alb S:U	S	Υ	α_2	β	r	p	SE %
1	3.92	12	86	3.7	0.008	-	0.995	0.001	4.6
2	3.54	48	86	5.4	0.02	-	0.995	0.001	4.7
3	3.25	96	75	15	0.04	-	0.997	0.001	3.6
4	2.93	10	36	7.8	0.06	-	0.999	0.001	0.6
5	2.65	128	57	10	0.14	-	0.999	0.001	4.0
6	2.61	20	100	7.8	0.19	-	0.986	0.01	6.1
7	2.49	0.4	70	15	0.21	0.015	0.999	0.001	2.2
8	2.15	7	70	13	0.62	0.04	0.997	0.001	3.8
9	1.91	2	88	22	1.4	0.10	0.996	0.001	3.6
10	1.85	28	88	29	1.9	0.12	0.995	0.001	5.8
11	1.77	6	86	19	1.9	0.15	0.994	0.001	6.1
12	1.64	7	117	29	3.2	0.28	0.992	0.001	6.0
13	1.54	3	71	5.3	3.1	0.20	0.944	0.01	17.4
14	1.42	4	100	50	3.1	0.81	0.999	0.001	1.7
15	1.20	20	83	50	5.2	1.5	0.999	0.001	1.4
16	1.13	72	75	32	10	1.3	0.984	0.001	5.4
17	1.10	28	54	40	5.6	0.8	0.992	0.001	4.4
18	1.05	12	100	60	8.6	2.7	0.997	0.001	2.0
19	0.80	100	82	60	18	5.1	0.993	0.001	2.3
20	0.66	68	96	54	20	9.6	0.997	0.001	1.2
21	1.44	520	80	59	3.1	-	0.986	0.01	4.0
22	1.08	600	83	60	17	1.5	0.965	0.01	7.3
23	0.87	400	86	74	15	4.9	0.993	0.001	2.4
24	0.79	460	80	58	14	-	0.999	0.001	0.5
25	1.45	2250	108	76	3.5	-	0.976	0.01	5.2
26	1.28	3200	72	42	4.2	-	0.999	0.001	1.0
27	1.17	2340	121	69	6.8	-	0.982	0.01	3.5
28	0.89	1790	98	68	15	4.4	0.994	0.001	2.3
29	0.78	6160	100	100	16	-	0.957	0.05	3.6
30	0.59	2560	100	84	27	13	0.994	0.001	1.5

ratios range from 7 - 21%, figures which agree with those of Soothill (1962). However, when these figures are translated into logarithms the values are much smaller (1.4 - 7.6%) and, as would be expected, the coefficient of variation of the selectivity value is even less (1.9 - 3.7%). The error involved when random urine collections were made, as opposed to 24 hour collections (5.3%), was slightly higher than the reproducibility of the method. However the random value was not systematically higher or lower and therefore when a 24 hour collection was not available a random one was always taken instead.

The apparent linearity of the log-log relationship was confirmed in 30 estimations of selectivity of varying values of -k and different degrees of proteinuria. Table 7 gives details of the relative clearances of individual proteins, selectivity (-k), the correlation coefficient, and the standard error of estimate of the individual protein clearances. The serum: urine albumin ratio is also given. It can be seen that during proteinuria the correlation coefficient is highly significant and even when the serum: urine albumin is over 1000 the correlation is still very significant. Very occasionally a break in the line was seen, when the clearance of one protein was exceptionally high or low, For example, in patient 13, the clearance of γ-globulin was found on every estimation of selectivity to be low, and the correlation coefficient was correspondingly less significant. Clearances of transferrin were sometimes higher than the albumin clearance, but this was not a consistent finding, and the value was rarely greater than 120%.

No immunochemical differences have been noted in specimens stored 8 days at 4°C without preservatives (Patte, Baldassaire and Loret, 1958) but proteins, especially lipoproteins, can undergo degradation on freezing and thawing (Lindgren and Nicols, 1960). Although estimations were generally

TABLE 8.

Stability of proteins by immunodiffusion after deep freezing

Estimations of the relative clearances of individual proteins - transferrin (S), Υ-globulin (Υ), α_2-macroglobulin (α_2) and β-lipoprotein (β) - were carried out before and after deep freezing for varying intervals of time. The degree of proteinuria, as indicated by the albumin serum:urine ratio (Alb S:U) and the index of selectivity (-k) are also given.

	Estimations on fresh samples						Time of deep freezing	Estimations after deep freezing					
Sample	Alb S:U	S	Υ	α_2	β	-k		Alb S:U	S	Υ	α_2	β	-k
1	160	84	50	35	<2.2	0.40	2 weeks	224	89	70	22	<2.8	0.61
2	7	88	22	9.8	5.6	0.79	"	6	100	38	9.6	2.8	1.00
3	5	50	42	4.9	4.6	0.88	"	8	67	57	7.2	<0.14	0.91
4	20	89	14	2.3	0.46	1.47	"	16	80	13	3.2	<0.4	1.37
5	1.75	156	44	1.5	0.39	1.71	"	0.875	91	23	1.4	0.19	1.78
6	0.875	88	25	0.71	0.10	1.98	"	1	100	25	0.78	0.10	1.99
7	16	80	29	15	12	0.57	3 weeks	16	50	29	17	15	0.46
8	5	84	44	2.2	1.1	1.41	"	5	71	42	3.1	-	1.40
9	12	67	50	4.1	2.4	1.09	4 weeks	8	80	50	6.7	0.26	1.56
10	24	86	11	1.5	0.21	1.73	"	16	57	7.2	1.3	<0.1	1.72
11	7	70	22	0.65	0.26	1.76	"	8	57	13	0.80	<0.09	1.84
12				11	6.4		4 months				34	<0.4	
13				11	3.8		"				18	2.7	
14					3.3		"					1.5	
15					1.0		"					0.07	
16					0.84		"					<0.04	
17				0.79	0.68		"				0.79	<0.09	
18					0.39		6 months					0.55	
19	16	66	16				7 months	24	43	19			
20	8	95	7.9				9 months	10	72	7.8			
21	3	53	5.9				"	2.5	50	3.9			

carried out within one, or at most two weeks, of collecting the specimens and before deep freezing, occasionally an estimation required to be repeated after storage at -20°C. The stability of the immunological activity of the proteins was therefore examined. Repeated freezing and thawing of serum and urine up to 10 times had no effect on the antigenicity of albumin or β-lipoprotein. However after deep freezing for 2 weeks, β-lipoprotein was often not detectable in the urine, and when it was detected the relative clearance was usually reduced. Presumably storage in a dilute solution affected the urine β-lipoprotein relatively more than the serum β-lipoprotein. The results are shown in Table 8. When a value for the clearance of β-lipoprotein was not obtained, the index of selectivity was calculated on the basis of 4, as opposed to 5, points; the differences were not significant. Selectivity was only affected when the β-lipoprotein clearance was reduced, and the value of the index of selectivity was then correspondingly raised. The immunological activity of the other four proteins was extremely stable and clearance values were not significantly affected after 4 months storage or more. Therefore, with the exception of β-lipoprotein, it was considered valid to repeat estimations after deep freezing.

In view of the fact that the index of selectivity was calculated from only 4 or 5 points, clearances of additional proteins were studied, in exactly the same way, namely orosomucoid, prealbumin, ceruloplasmin, γ_{1A}-globulin, α_1-lipoprotein and fibrinogen. This was in order to see if a change in selectivity or a greater sensitivity was obtained using a larger range of proteins. The molecular weights of the proteins are given in Table 4, and suitable antibody and antigen concentrations were worked out by a systematic trial, as for the other proteins. The dilutions used are shown in Table 9. Immunoelectrophoresis (2.3.2) was used to check the specificity

TABLE 9

Antisera, serum and urine dilutions used in estimating relative clearances of six other individual proteins.

ANTISERA TO:	SERUM DILUTIONS				URINE DILUTIONS		ANTISERA DILUTIONS					
Prealbumin	1/8	1/16	1/32	1/64	2	1	1/10	1/5	3/10	2/5	1/2	3/5
Orosomucoid	1/4	1/8	1/16	1/32	2	1	1/5	3/10	2/5	1/2	3/5	7/10
Ceruloplasmin	1/8	1/16	1/32	1/64	2	1	1/5	3/10	2/5	1/2	3/5	7/10
γ_{1A}-Globulin	1/8	1/16	1/32	1/64	2	1	1/10	1/5	3/10	2/5	1/2	3/5
α_1-Lipoprotein	1/8	1/16	1/32	1/64	2	1	1/5	3/10	2/5	1/2	3/5	7/10
Fibrinogen	1/32	1/64	1/128	1/256	2c	c/2	1/20	1/15	1/10	1/6	1/4	1/2

The urine dilutions are given for an Albustix concentration of 300 mg.%. Stronger or weaker dilutions were used for lower or higher protein concentrations. Plasma was used instead of serum for fibrinogen estimations.

c = urine concentrate.

of the antisera. The anti-orosomucoid serum was found to have two components and some experiments were carried out using gel filtration and neuraminidase, in order to identify the orosomucoid.

The determination of selectivity by immunodiffusion was found to be expensive, but it was a quick method requiring only small samples of serum. As many as six clearances could be set up per day. Although the individual errors are high, the overall error of the method is not more than 4%. Inaccuracy in reading the plates and diluting the specimens probably contribute equally to this error.

The selectivity of the proteinuria of all the patients in the study was estimated at regular intervals by immunodiffusion.

2.3.2. IMMUNOELECTROPHORESIS

Immunoelectrophoresis is a combination of electrophoresis and immunodiffusion; protein is separated electrophoretically into albumin and the globulin fractions before the antisera is added. Double diffusion of antibody and separated antigens then takes place and numerous arcs of precipitation can form, each arc being specific for an individual protein. This method is only a qualitative one, but it gives a useful overall picture of the number of different components in a mixture of proteins. Direct comparison of serum and urine proteins can be made, and by relating the proteins to their molecular weights (Table 4) indication of the selectivity can be obtained. Immunoelectrophoresis was carried out on serum and urine samples from some patients with varying degrees of protein selectivity and with unusual excretion patterns.

A modification of the method of Scheidegger (1955) was followed, using LKB equipment. This enabled as many as 18 immunoelectrophoretic separations to be set up at the one time.

Special slide trays containing 6 microscope slides (7.5 x 2.5 cm.) were placed on a levelling table. Agar, 20 ml. of 1.5% w/v, in 0.05 M barbitone buffer pH 8.6, was poured on to each slide tray and the agar was allowed to cool. The slide tray was transferred to a moist chamber for 18 - 24 hours. A template was then used to cut sample application holes and an antisera trough in the gel; the samples were applied and the slide tray was placed in position for electrophoresis. The electrode compartments contained 0.1 M barbitone buffer pH 8.6; connection to the gel was made by chamois leather wicks soaked in 0.05 M buffer. A current of 7.5 mA/slide was passed for 1.5 - 2 hours, after which the antisera (Behringwerke or Hyland) was applied to the troughs and the slide trays returned to the

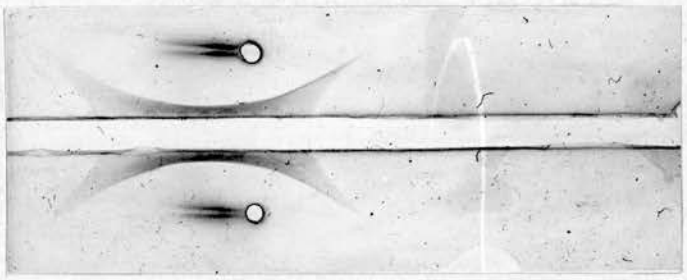
TABLE 10.

Stains used in immunoelectrophoresis

Stain	Specificity	Reference
Ponceau S	All proteins	Smith (1960)
Amidoschwartz	All proteins	Smith (1960)
Sudan black in ethanol	Lipoproteins	Swahn (1960)
O-dianisidine in acetate buffer + hydrogen peroxide	Haptoglobin	Smith (1960)
P-phenylene diamine in acetate buffer	Ceruloplasmin	Uriel (1964)
Periodic acid + α naphthol and p-phenylene diamine + hydrogen peroxide	Glycoproteins	Uriel (1964)

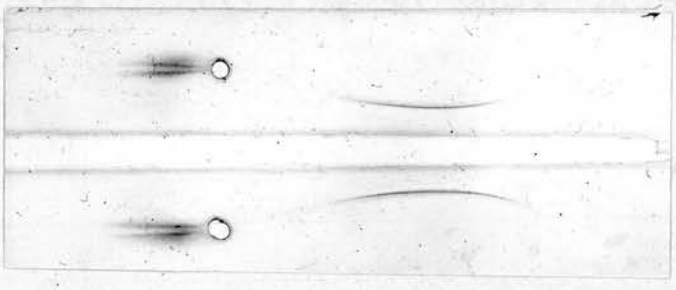
Fig. 11. Identification of individual proteins by immunoelectrophoresis.

1. Serum reacted against anti-albumin



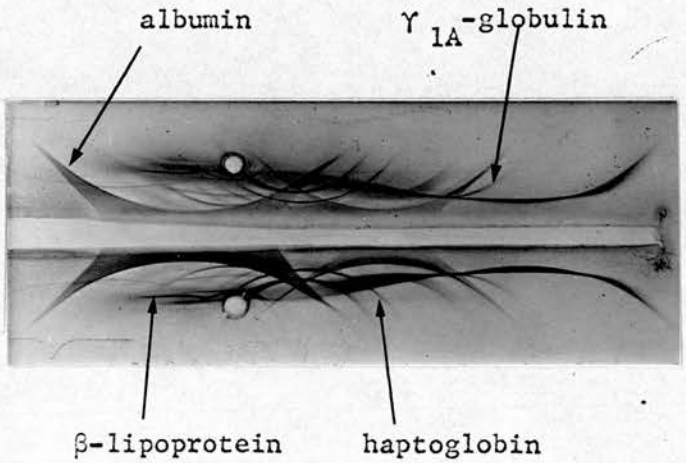
stained by
Ponceau S

2. Serum reacted against anti-γ_{1A}-globulin



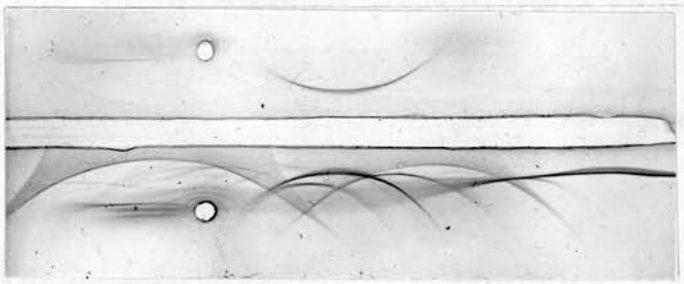
stained by
Ponceau S

3. Serum reacted against anti-human serum



stained by
Ponceau S

4. Serum reacted against anti-human serum



stained for lipoprotein
and haptoglobin

stained by
Ponceau S

moist chamber for 48 hours. Excess protein was removed by washing with 0.9% w/v NaCl, for 48 hours, the saline being changed at 24 hours. Strips of filter paper were then placed on the surface of the agar to absorb the salt and the preparation was dried. The filter paper was easily removed after soaking with water, and the slides were stained and viewed on a photographic enlarger. The different stains used are detailed in Table 10.

The serum and urine protein separations for selectivity studies were generally reacted against anti-human serum (Hyland), when numerous precipitin arcs were seen. The individual proteins were identified by means of specific antisera (Behringwerke) or special stains or by comparison with separations described in the literature (Peetoom, 1963; Grabar and Burtin, 1964). Fig. 11 demonstrates examples of the identification of some of the individual proteins. Serum was reacted immunoelectrophoretically against (1) anti-albumin, (2) anti-γ_{1A}-globulin, (3) anti-human serum and (4) anti-human serum. Preparations 1 - 3 were stained with Ponceau S, preparation 4 was stained for haptoglobin and lipoprotein. By comparison of slide 3 with all the other slides it was possible to identify in the mixture of serum proteins the precipitin arcs of the 4 individual proteins (albumin, γ_{1A}-globulin, haptoglobin, β-lipoprotein).

The urine was concentrated (2.2.2) before immunoelectrophoresis, to contain a minimum of 5 g.% protein. Effluents from column chromatography (2.3.3) which were examined immunoelectrophoretically were also first concentrated.

In addition to using the specific antisera to identify the precipitin arcs, the specificity of the antisera used in the immunodiffusion studies (2.3.1) was checked by immunoelectrophoresis.

2.3.3. GEL FILTRATION

Gel filtration has been increasingly used since its introduction in 1959, and is now accepted as the most convenient and satisfactory method for fractionation according to molecular size. Sephadex is a cross-linked dextran, which in water forms a swollen gel with molecular sieving properties. Large molecules are excluded from the body of the gel and smaller molecules are partially or completely retained within the gel particles according to their molecular size. If a mixture of components is subjected to column chromatography on Sephadex the elution volume of each component is therefore in an inverse relationship to molecular radius. The theory and applications of gel filtration are reviewed by Flodin (1962), Ackers (1964), Laurent and Killander (1964) and Squire (1964).

The relationship of elution volume (or tube number) and logarithm of molecular weight on Sephadex G 200 is approximately linear over the molecular weight range 20,000 - 600,000 (Andrews, 1965). By comparing urine and serum protein concentrations within this molecular weight range, for corresponding tube numbers, relative renal clearance of protein in relation to molecular weight, and hence selectivity, can be calculated.

Details of the method

The method of determination of selectivity of proteinuria by gel filtration was an adaptation of that of Hardwicke (1965). Two columns were employed, one 100 x 2 cm., the other 60 x 2 cm. The Sephadex G 200 was soaked in distilled water, with added toluene as an antibacterial agent, for at least one month, since prolonged swelling has been shown to extend separation towards the high molecular weight range (Andrews, 1965). The fines were then exhaustively removed by decantation. The gel was given a final washing with a tris buffer (0.1 M tris + 0.1 M NaCl, pH 8.0, with 0.01%

w/v sodium azide to prevent bacterial growth). The column was then packed and operated at a pressure head of 5 - 25 cm., with the tris buffer as eluant.

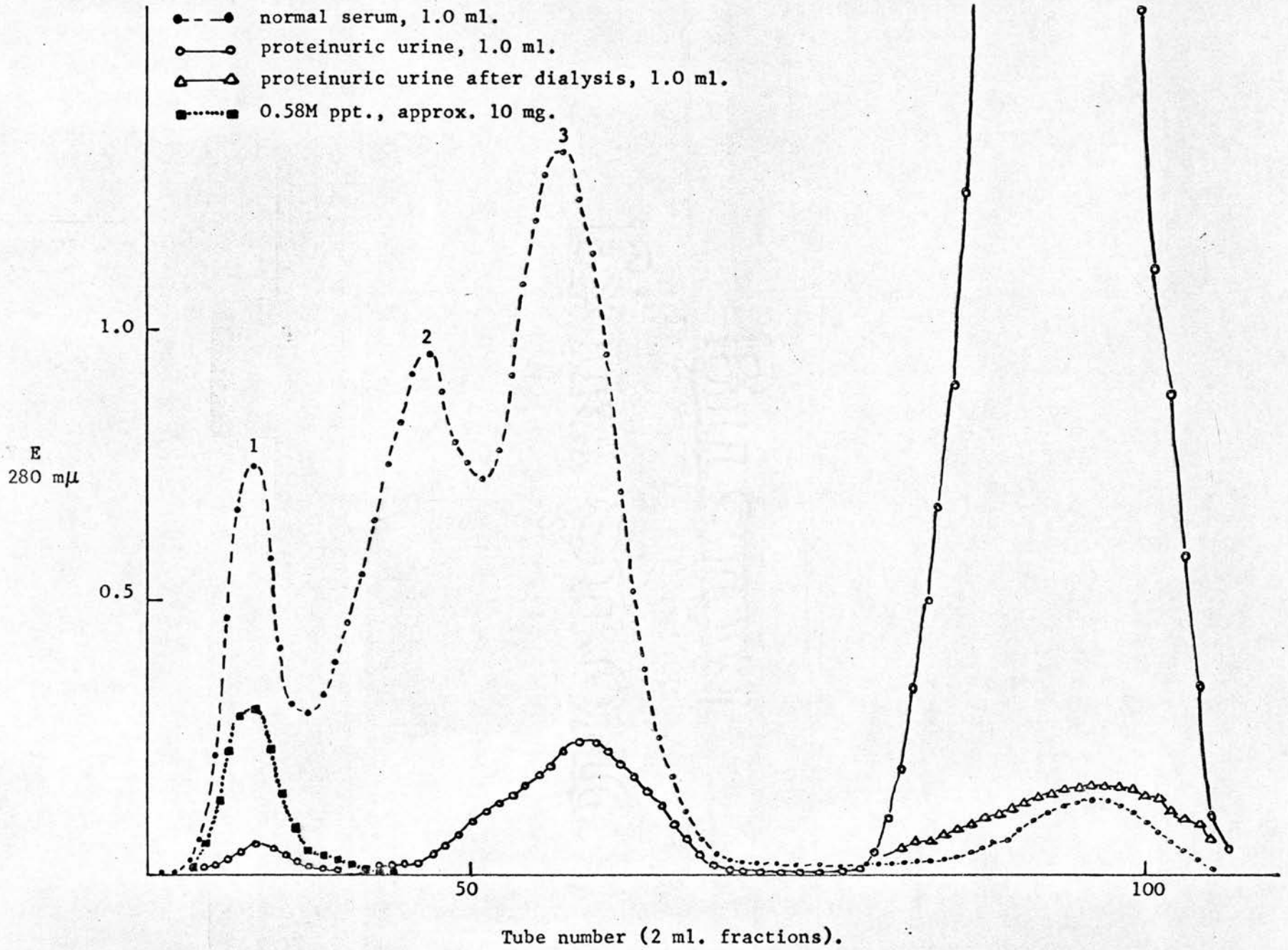
It was found that a newly packed column would give good flow rates of up to 15 ml. per hour at a pressure head of 5 cm.. Over a period of weeks the gel packed more tightly and the pressure head required to be increased to maintain a good flow. A satisfactory column could be operated for about six weeks before it needed repacking. The separation of proteins was found to be less satisfactory with Sephadex that had been used for more than 4 to 6 months. When the degree of separation deteriorated the Sephadex was discarded and a new batch was used.

An aliquot of serum (0.5 - 2 ml.) and subsequently a similar volume of the corresponding urine, concentrated to contain approximately the same amount of protein, was applied to the column. Application was made by layering the sample under the buffer, immediately above the Sephadex. Constant volume aliquots of eluant were obtained using siphons of 3 ml. capacity for the larger column, and 2 ml. for the smaller, in conjunction with an LKB fraction collector.

Serum is separated into three peaks. The first peak eluted contains material of sedimentation constant 19S, with some 11S; the second peak contains 7S material and the third peak contains 4S material (Killander and Flodin, 1962). Urine is similarly separated, although the distribution is different. The different fractions were examined by immunoelectrophoresis, to identify the serum and urine proteins contained in each peak. A small peak, with extinction at 280 mμ and eluting long after the protein, is found on gel filtration of serum. A much larger peak, eluting at about the same point is found on gel filtration of urine (Fig. 12). The urine peak is

Fig. 12. Gel filtration of serum, proteinuric urine, and 0.58M precipitate.

Proteins are separated into three peaks by Sephadex G 200; peak 1 contains 19S material, peak 2 7S and peak 3 4S.



largely removed by dialysis, but in order to remove all low molecular weight substances which might give extinction readings at 280 mμ the column was washed with at least two elution volumes of buffer between samples and the effluent was then checked at 280 mμ.

Concentration of the urine was achieved by the use of PEG (2.2.2). The urine concentrate was dialysed against distilled water for 24 hours, brought to 0.58 M by the addition of 3.5 M NaCl, dialysed against 0.58 M NaCl and then centrifuged before being applied to the column. This adjustment of tonicity precipitates Tamm-Horsfall protein, a high molecular weight urinary tract protein comprising about 70% of the total urinary tract proteins (Maxfield, 1961). The 0.58 M precipitate was dissolved in alkaline distilled water and subjected to gel filtration when it was found to elute with the 19S material (Fig. 12).

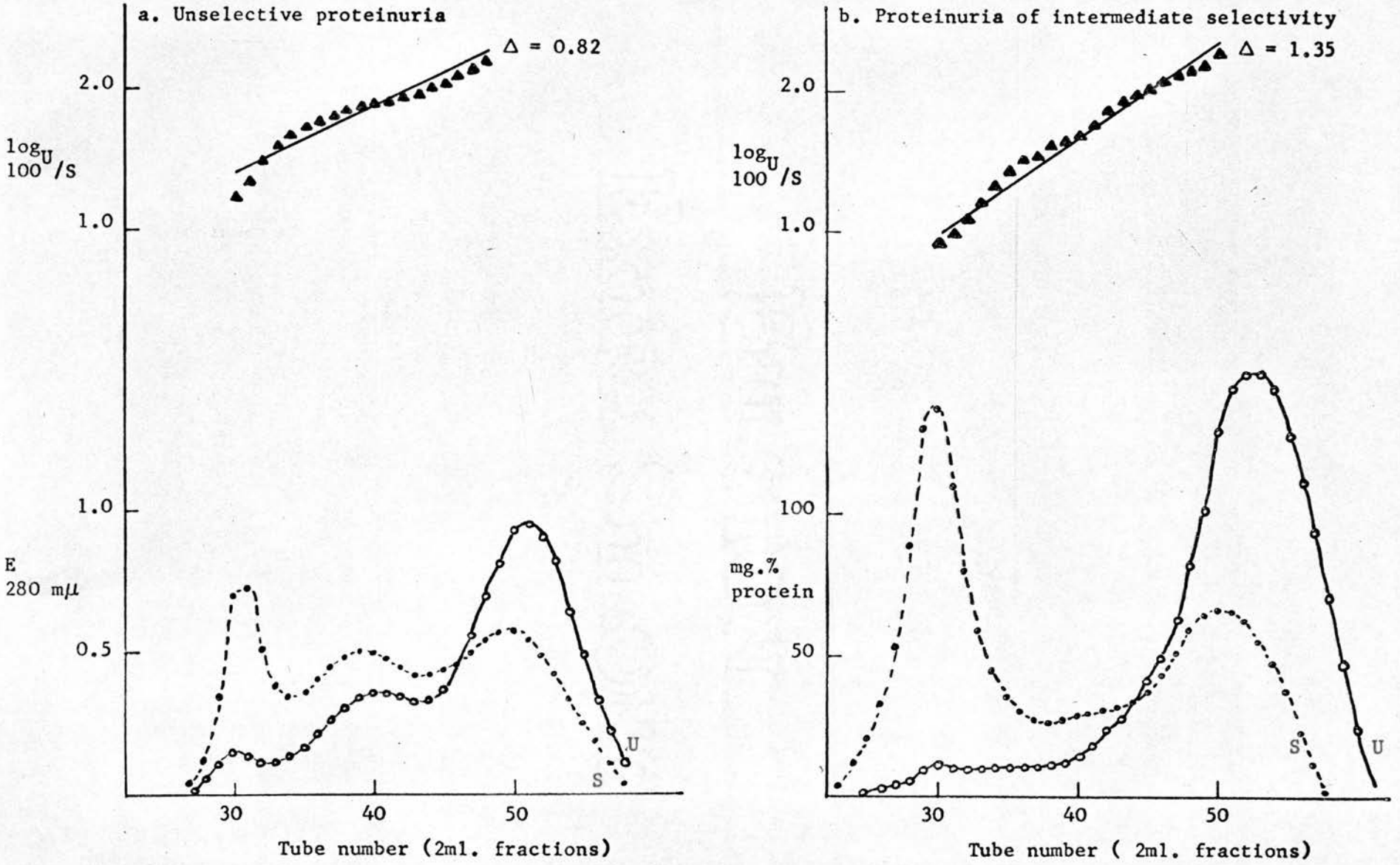
The dialysis and centrifugation subsequent to concentration are important, as some polyethylene glycol is known to cross the dialysis membrane and could theoretically affect the protein estimations of the column effluents. This problem has already been discussed (2.2.2). In order to assess any affect of counter-dialysed polyethylene glycol on the accuracy of the results some of the urines were also concentrated by pressure ultrafiltration (2.2.2). The protein concentrations of the effluent fractions from the column were determined by reading the extinction at 280 mμ or by a modified Folin-Ciocalteau method using an AutoAnalyzer (2.2.1).

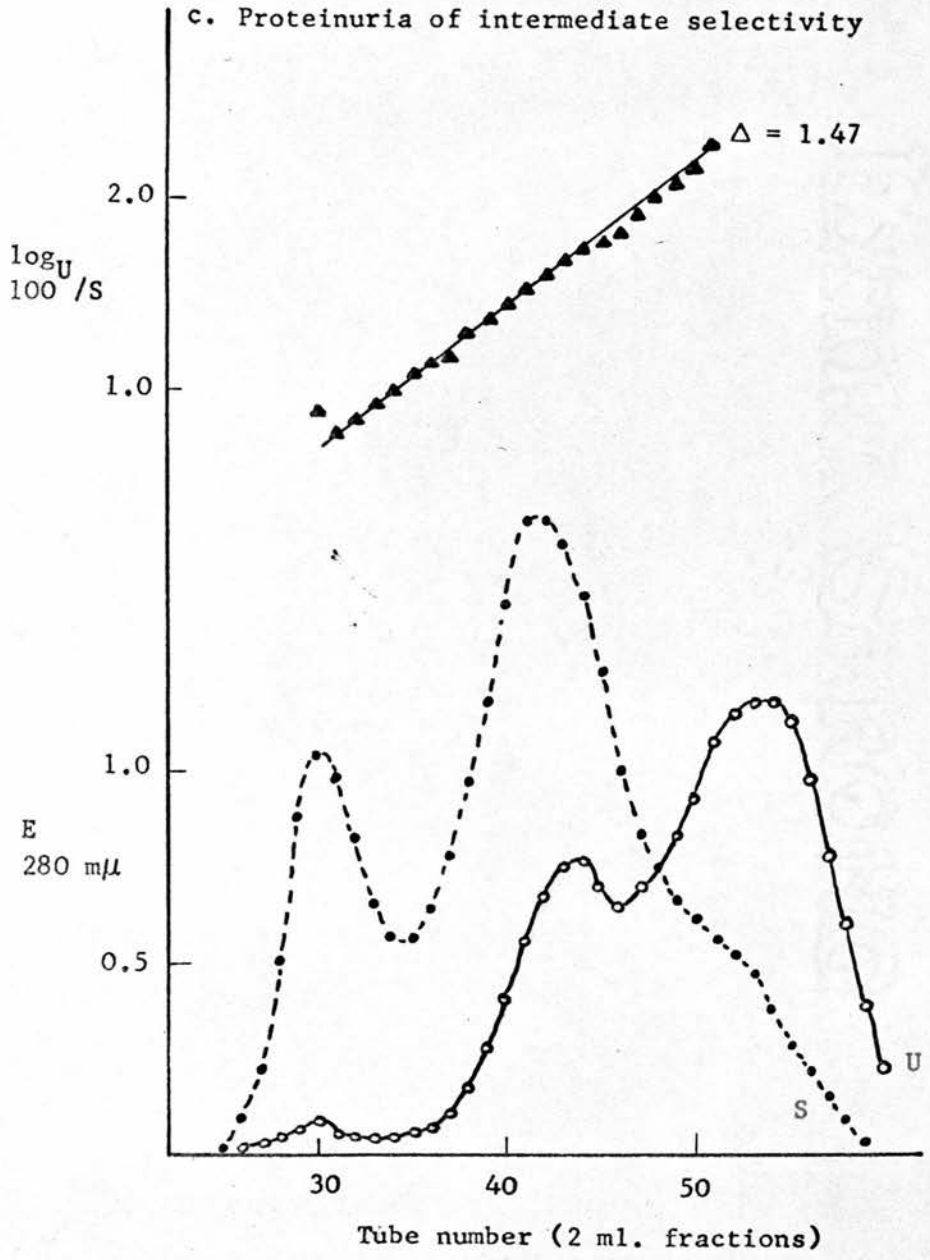
Calculation of selectivity

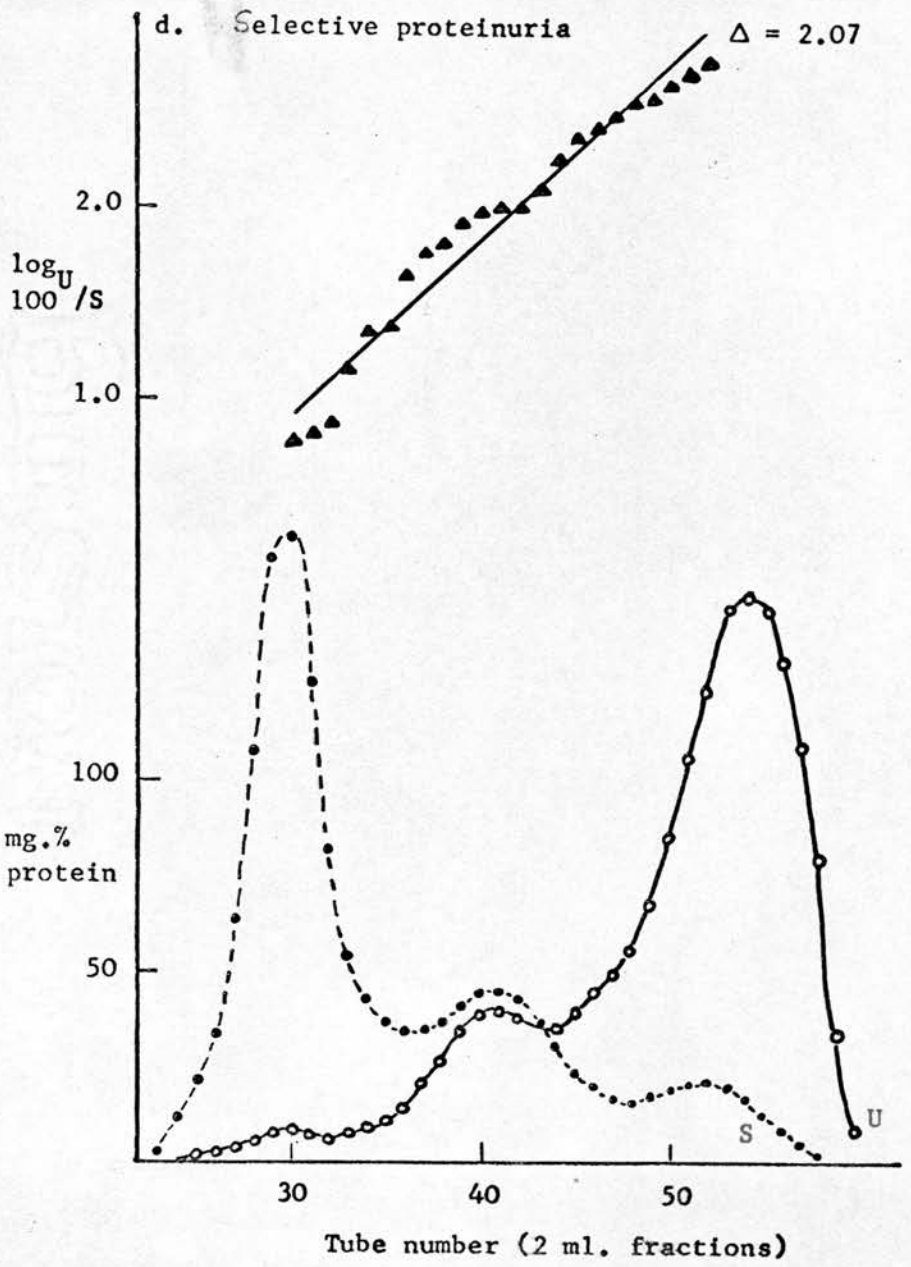
The protein concentrations of the serum eluates and the corresponding urine eluates were plotted against tube numbers. The urine:serum ratio for each tube number was calculated and (for ease of subsequent calculation) multiplied by 100. The logarithm of :-

Fig. 13. Determination of selectivity of proteinuria by gel filtration.

Typical serum (S) and urine (U) protein separations on Sephadex G 200 and plots of $\log_{10} 100\ ^{U}/_{S}$ against the tube number. The index of selectivity (Δ) is the total change in relative clearance of protein over a fixed molecular weight range.




c. Proteinuria of intermediate selectivity
Δ = 1.47
2.0
1.0
log U/S
100
1.0
0.5
E
280 mμ
U
S
30
40
50
Tube number (2 ml. fractions)


d. Selective proteinuria
Δ = 2.07
2.0
1.0
log U/S
100
100
mg.%
protein
50
U
S
30
40
50
Tube number (2 ml. fractions)

$$\frac{\text{100 x urine concentration (U)}}{\text{serum concentration (S)}}$$

was then plotted against tube number. Since protein separation on Sephadex is proportional to molecular weight, the resulting graph showed relative clearance of protein against molecular weight on a log-log scale. The relationship was approximately linear over the range of effective protein separation on G 200. The slope of the line was steep when the proteinuria was selective, and much flatter when the proteinuria was unselective.

Using the method of least squares the slope of the regression line relating $\log_{10} 100^{U}/S$ to tube number was calculated, using the points lying between the first and third serum protein peaks, as G 200 is known to resolve well within this area (Andrews, 1965). In order to obtain an index of selectivity which was independent of column size and volume of effluent fractions the total change in $\log_{10} 100^{U}/S$ between the first and third serum peaks was taken. This index (Δ) was calculated by multiplying the slope of the line by the number of tubes between the apex of the first serum peak and the apex of the third serum peak. Fig. 13 shows typical serum and urine separations on G 200, the plots of $\log_{10} 100^{U}/S$ against tube number and values of Δ. It can be seen that serum as well as urine patterns can show great variation and gel filtration can often give valuable information about a patient's serum protein distribution.

Errors of the method

The accuracy and possible errors of the method were investigated in several ways.

Recovery of protein in five experiments ranged from 101 - 105% with a mean of 102.6%. The index of selectivity (Δ) was determined five times

TABLE 11.

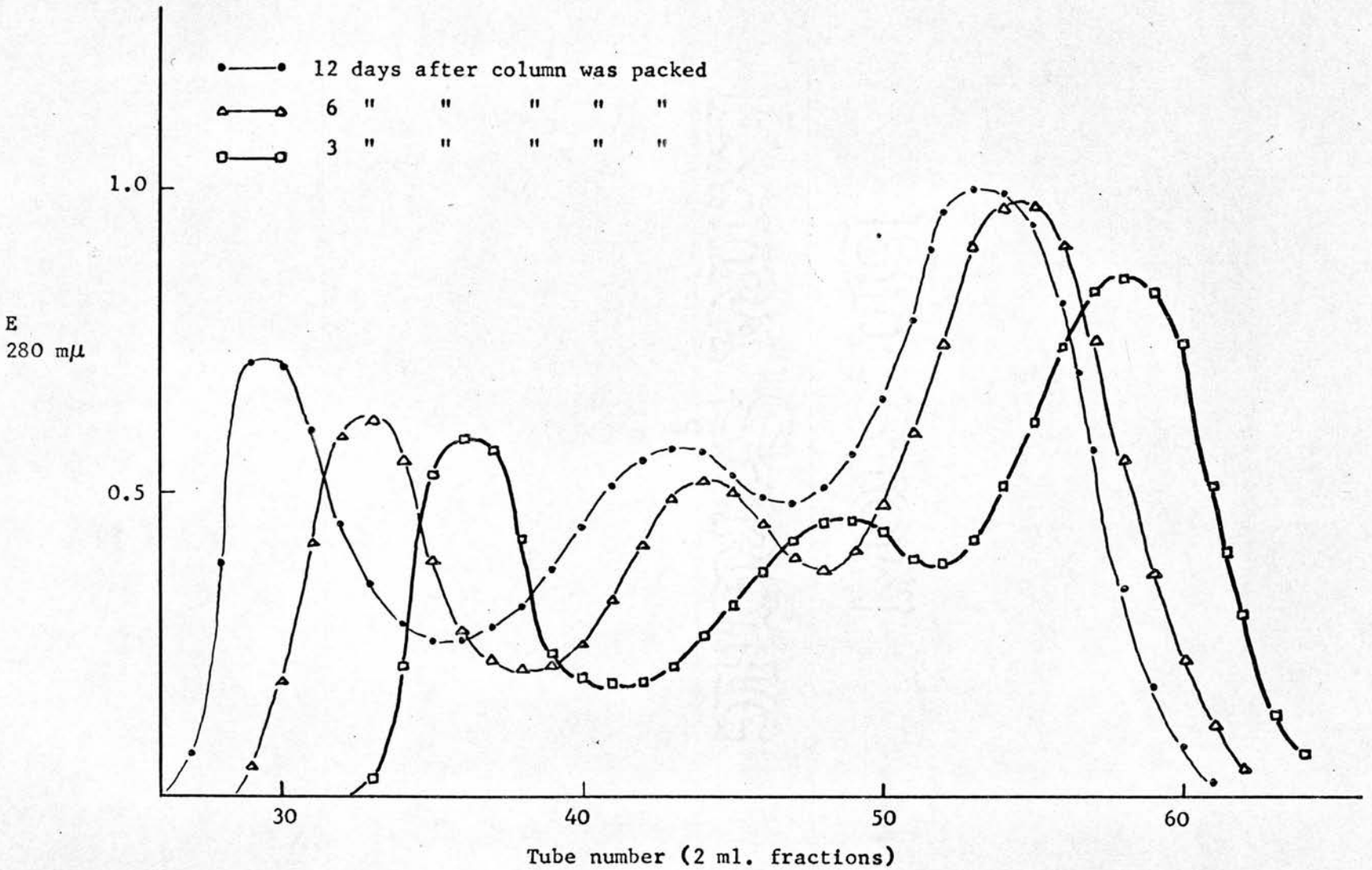
The linear relationship between relative clearance and molecular weight of protein on a log-log plot by gel filtration

The correlation coefficient (r), its limits of significance (p) and the standard error of estimate of log 100 urine/serum (SE) were estimated in 18 subjects with varying values of the index of selectivity (Δ). Proteinuria was over 1 g./day in 12 subjects and under 1 g./day in 6 subjects.

Subject	Δ	r	p	SE %
Proteinuria over 1 g./day				
1	2.51	0.975	0.001	12.3
2	2.29	0.968	"	9.4
3	1.92	0.995	"	2.9
4	1.84	0.973	"	8.6
5	1.66	0.978	"	4.4
6	1.43	0.973	"	4.5
7	1.32	0.992	"	5.1
8	1.20	0.979	"	4.7
9	1.08	0.997	"	2.4
10	1.02	0.924	"	7.3
11	0.95	0.995	"	2.5
12	0.89	0.991	"	1.6
Proteinuria under 1 g./day				
1	1.70	0.976	"	6.3
2	1.15	0.991	"	2.9
3	0.93	0.968	"	4.1
4	0.64	0.766	"	12.1
5	0.54	0.857	"	7.2
6	0.39	0.888	"	3.1

Fig. 14. Variation of gel filtration elution patterns.

Normal serum (0.5 ml.) on Sephadex G 200.



on one serum and the corresponding urine in order to determine the reproducibility of the estimation. Five aliquots of serum and five aliquots of urine were passed through the column and these five pairs of separations were used to calculate Δ. Values ranged from 1.15 to 1.30, mean = 1.22, S.D. $\pm$ 0.07, coefficient of variation 5.8%. A series of sixteen duplicate determinations of Δ gave a coefficient of variation of 6.9%.

The apparent linear relationship of $\log_{10} 100^U/S$ and tube no. was found in 18 experiments to have highly significant correlation coefficients. The results are shown in Table 11 which also gives the standard error of estimate of the values of $\log_{10} 100^U/S$. The correlation is less significant in cases where the total urine protein was under 1 g./day.

The elution pattern from a Sephadex G 200 column was found to change with time (Fig. 14), particularly when a column was newly packed. There was, however, practically no change between successive runs. Since a urine concentrate was nearly always run immediately after the corresponding serum, changes in column characteristics did not contribute appreciably to errors in the estimation of Δ. As a rule, the tubes of serum eluate could be aligned with the corresponding tubes of urine eluate on the basis of the tube number. Correct alignment was indicated by the fact that the first urine protein peak coincided with the first serum protein peak. Occasionally the first serum and urine peaks were found to be separated by one or at the most two tubes; this was probably due to irregular emptying of the siphon. The urine tubes were then renumbered to make the first urine peak coincide with the first serum peak. With patients who had a very selective proteinuria this adjustment could not easily be made, as the amount of urinary protein in the early tubes was small and a definite first peak was not always detected. Because of this occasional difficulty in matching the

TABLE 12.

Effect on protein selectivity of shifting urine elution patterns

Values of selectivity (Δ) calculated from elution patterns in which the 1st peaks of serum and urine are at the same tube number compared with values calculated when the urine plot is shifted 1 or 2 tubes to the left. This shift tends to make the 3rd peaks of serum and urine coincide. Δ is changed by similar amounts but to lower values when the urine plot is shifted to the right.

Peak 1 Matching	1 tube moved	% error	2 tubes moved	% error
0.68	0.72	5.9	0.75	10.0
0.86	0.92	7.3	0.95	10.0
1.01	1.14	13.0	1.21	20.0
1.26	1.34	2.6	1.40	3.3
1.52	1.56	3.5	1.57	8.2
1.71	1.77	3.1	1.85	6.7
1.92	1.98	6.3	2.05	11.0

effluent patterns, it seemed important to estimate the errors involved when selectivity was calculated from deliberately misplaced plots. Table 12 gives values of Δ over a range of selectivity, calculated from plots shifted by one tube and by two tubes. An alignment error of ± one tube varies the calculated Δ by a mean of 6%, which is of the order of magnitude of the overall reproducibility of the method; an error of two tubes varies it by a mean of 10%.

Lining up of the serum and urine patterns on the basis of the third protein peak is not a sound procedure. Because of the relatively high clearance of low molecular weight protein, the urine generally contains a relatively large proportion of proteins with a molecular weight less than that of albumin (69,000), whereas these small molecular weight proteins constitute a very small proportion of the serum protein. The composition and average molecular weight of the proteins eluted in the third urine peak are therefore different from those of the third serum peak, and the urine peak is usually eluted two or three tubes after the serum peak.

Nephrotic sera tend to be lipaemic and occasionally the elution of large amounts of lipid into the early tubes of serum eluate gave a spuriously high first protein peak at 280 mμ which was due to turbidity. This difficulty could often be alleviated by obtaining fasting blood. When the serum eluate was turbid, however, reading the protein by the Folin-Ciocalteau method rather than at 280 mμ gave more accurate values for the protein concentrations.

Although the amount of urinary protein applied to the column was generally similar to the amount of serum protein, occasionally too much or too little urinary protein was mistakenly added. To investigate the error involved, a series of selectivity values were calculated from experiments

Fig. 15. Effect on selectivity of overloading and underloading the column with urine protein.

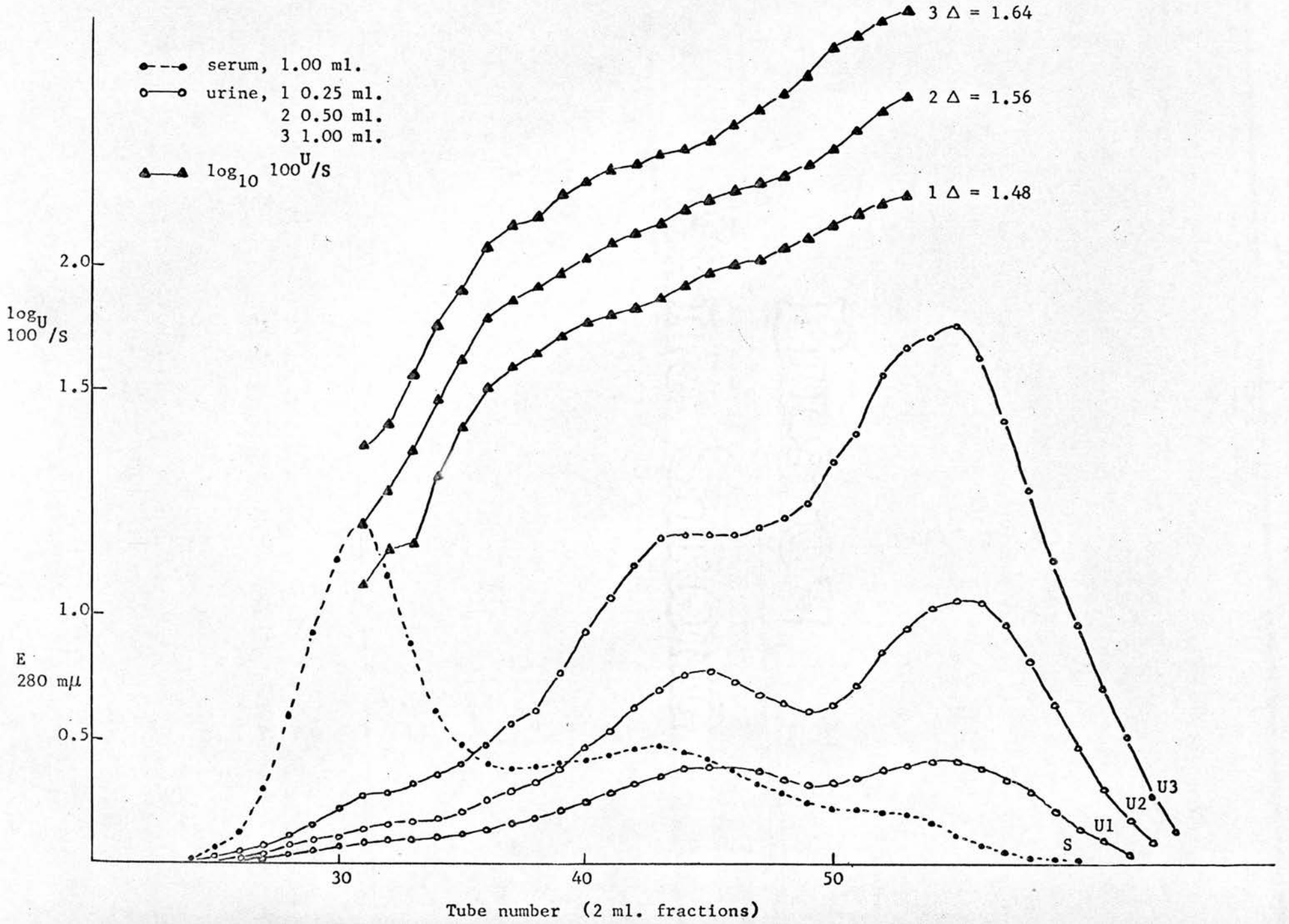


TABLE 13.

Stability of proteins by gel filtration after deep freezing

Indices of selectivity (Δ) were determined before and after deep freezing for varying time intervals.

Specimen	Initial Δ	Time of deep freezing	Repeat Δ	% error
1	1.92	4 months	3.32	73
2	1.02	2 months	1.13	11
3	1.61	6 weeks	1.62	0.7
3	1.61	3 months	2.20	37

TABLE 14.

Selectivity values estimated by gel filtration using different methods of estimation and concentration of protein

Comparison of values of selectivity (Δ) obtained when the urine is concentrated by polyethylene glycol (PEG) and ultrafiltration (UF) and when the effluent proteins are estimated by reading the extinction at 280 mμ or by a modified Folin reaction.

Subject	Δ PEG				Δ UF				Mean PEG	Mean UF	Difference (PEG-UF)	% Difference (PEG-UF)
	280 mμ	Folin	Difference (280 mμ - Folin)	% Difference (280 mμ - Folin)	280 mμ	Folin	Difference (280 mμ - Folin)	% Difference (280 mμ - Folin)				
1	1.22	1.35	+0.13	+10.7								
2	1.05	1.16	+0.11	+10.7								
3	2.00	2.16	+0.16	+ 8.0								
4	1.34	1.29	-0.05	- 3.7								
5					0.99	1.15	+0.16	+16.2				
6	0.80	0.86	+0.06	+ 7.5	0.77	0.81	+0.04	+ 5.2	0.83	0.79	-0.04	- 4.8
7	1.05	1.16	+0.11	+10.5	0.95	0.97	+0.02	+ 2.1	1.10	0.96	-0.14	-12.7
8	1.34	1.35	+0.01	+ 0.7	1.47	1.53	+0.06	+ 4.1	1.35	1.50	+0.15	+11.1
9	1.92	2.16	+0.24	+12.5	2.07	2.27	+0.20	+ 9.7	2.04	2.17	+0.13	+ 6.4
10	2.08	2.04	-0.04	- 1.9	2.31	2.07	-0.24	-10.4	2.06	2.19	+0.13	+ 6.3
Mean	1.42	1.50	+0.10	+ 6.1	1.43	1.47	+0.04	+ 4.5	1.48	1.52	+0.05	+ 1.3

where the column was deliberately overloaded and underloaded. The results are shown in Fig. 15. Both overloading and underloading gave a variation in Δ of 5.1%, a value which is within the reproducibility of the method.

The stability of the molecular size distribution of serum and urinary proteins after deep freezing was tested by estimating selectivities before and after storage at -20°C. The results are shown in Table 13. Selectivity values are affected after storage of six weeks. As might be predicted, the selectivity index changed towards higher values, presumably due to degradation of the high molecular weight urinary proteins, especially the lipoproteins (Lindgren and Nicols, 1960). Degradation is more significant in dilute solutions and urine will therefore be more affected than serum. Estimations of selectivity were generally carried out within one, or at the most two weeks, of collecting the specimens; however, on the few occasions when a column was not immediately available the samples were stored at -20°C before gel filtration, for a maximum period of one month.

Selectivities calculated on the basis of measurement of protein at 280 mμ were compared with those calculated on the basis of measurement of protein by the modified Folin-Ciocalteau reaction. Selectivities estimated when the urine protein was concentrated by PEG and ultrafiltration were also compared. The results are shown in Table 14. Measurement of protein by the Folin-Ciocalteau reaction tended to give consistently higher values of selectivity than those obtained when the effluent protein was estimated at 280 mμ. The mean overall difference, however, was 5.3%, an amount comparable to the reproducibility of the method. Although the use of PEG could theoretically introduce small errors into the effluent urine protein values, this was not found to be significant. The mean difference in selectivity between the concentration methods was only 1.3%

The coefficient of variation of the gel filtration method of estimating the selectivity of proteinuria (7%) was somewhat higher than that of the immunodiffusion method (4%), but gel filtration was found to be technically simpler, and less expensive. However, it was not suitable for numerous estimations, since a single determination, using one column, took about 48 hours. For this reason gel filtration was not performed in every case, but about 35% of the patients studied had one estimation of selectivity carried out by this method.

TABLE 15.

Some properties of the enzymes studied

Enzyme	Principal sources	Molecular weight	Stability	Inhibitors	References
Amylase	Pancreas Also in saliva, urine, faeces, blood.	45,000	At least 1 week at R.T. or 4°C.	No known inhibitors in urine.	Abderhalden (1961) Saxon et al. (1957)
Pepsin(ogen)	Gastric mucosa Also in blood, urine, CSF.	48,000 activated to 34,000	Few days at R.T. 2 weeks at 4°C 3 weeks at -10°C.	No inhibitors found in urine.	Wilkinson (1962) Mirsky et al. (1948) Edwards, Jepson & Wood (1962)
LD	Most body tissues, including the kidney and blood.	136,000	Stable in frozen fluids, unstable in aqueous soln.	Dialysable inhibitor in urine.	Wilkinson (1962) Abderhalden (1961) Apella & Markert (1961)
GOT	Most body tissues including the kidney and blood.	110,000	3 weeks at 4°C.	? inhibitor in urine.	Dixon & Webb (1964) Wilkinson (1962)

2.3.3. ENZYMES

Many enzymes thought to be primarily of serum origin occur in the urine, and in renal disease the urinary concentration is often raised (Kemp & Laursen, 1960; Coltori et al., 1963; Szasz et al., 1965). In view of the relationship, in renal disease, between clearance of protein and molecular weight it was decided to investigate the clearance of some serum enzymes of known molecular weight, and to compare the results of the enzyme studies with those of the other proteins.

The serum enzymes were briefly surveyed. Some have awkward assay methods, the molecular weight of others is not known, and some are known to exist in a different molecular form in the urine. Finally four enzymes which did not possess any of these features were chosen. Two are known to be also present in renal tissue, lactic dehydrogenase and glutamic oxaloacetic transaminase, and two are thought to be absent from renal tissue, amylase and pepsinogen. The sources and molecular weights and other relevant details are shown in Table 15. It was hoped that a comparison of the clearances of enzymes present and absent in renal tissue might reveal any contribution of the kidney to the urinary concentration of the enzyme.

The enzyme concentrations in sera and corresponding urine were estimated, in most cases within 24 hours, and in all cases within 48 hours, of collecting the specimens. Sodium azide had no effect on any of the enzyme assays. Clearances were calculated in order to compare the values found with those predicted from selectivity data. Extinction measurements were made in an SP 500 or an SP 600 Unicam spectrophotometer.

Amylase

There are numerous methods of measuring amylase, and all have certain disadvantages. A modification of the method of Van Loon (Caraway, 1960)

was chosen for its speed and convenience. The coefficient of variation, although large, was found to be comparable to the coefficients of variation involved in estimating individual protein clearances by immunodiffusion.

Starch was incubated with serum or urine and iodine was then added. The intensity of the blue starch-iodine colour was compared to that of a control at 660 mμ. A calibration curve, using a serum with a high amylase content, showed linearity when the starch substrate was in excess. The units of amylase/100 ml. were defined as

$$\frac{\text{extinction (control-test)}}{\text{extinction control}} \times 800$$

The reproducibility of the method was investigated by estimating a serum and corresponding urine amylase 5 times each. The coefficient of variation for the serum estimation was 13%, for the urine estimation it was 9% and for the urine:serum ratio it was 22%. Since the coefficient of variation of the method was so high, all the amylase assays were performed in duplicate. No significant difference in amylase value could be detected in dialysed and untreated urines.

Pepsinogen

Pepsinogen was assayed as pepsin by modifications of the methods of Hunt (1948) and Edwards, Jepson and Wood (1962). The samples of plasma and urine were incubated at pH 2.0 with a human plasma substrate for 24 hours. Incubation was stopped by trichloracetic acid, and after centrifugation Folin-Ciocalteau reagent was added to the supernatant. The intensity of the blue colour was read at 680 mμ, 25 - 35 minutes after mixing the reagents. Two controls were used to estimate (1) the amount of "tyrosine-like" compounds present prior to incubation and (2) the amount of

"tyrosine-like" compounds formed by incubation of the plasma substrate alone. The amount of "tyrosine-like" compounds formed by incubation of the plasma or urine alone was found by subtraction. Calibration is generally by a phenol standard in conjunction with a dilution curve or by a series of tyrosine standards. Both methods were used although neither was found to be ideal. The phenol standard colour was appreciably less stable than the sample or tyrosine standard colour. The gastric juice dilution curve was not very satisfactory and the tyrosine standards, although linear, were not related to the peptic activity curve.

The reproducibility of the method was investigated by estimating a plasma and corresponding urine pepsin 5 times each. The coefficient of variation for the plasma estimation was 3.6%, for the urine estimation was 8.3% and for the urine:plasma ratio was 5.6%.

Lactic dehydrogenase

Lactic dehydrogenase (LD) was estimated by the method of Wroblewski and La Due (1955). Serum or urine was incubated with $NADH_2$ and pyruvate in phosphate buffer pH 7.5 and the rate of formation of NAD followed at 340 mμ. The unit of LD is defined as an extinction change of 0.001 per minute. All the urine samples were dialysed for a minimum period of two hours before the assay.

The reproducibility of the method was investigated by estimating a serum and corresponding urine LD 5 times each. The coefficient of variation for the serum estimation was 3.2%, for the urine estimation was 7.7% and for the urine:serum ratio was 10.3%.

Glutamic oxaloacetic transaminase

Glutamic oxaloacetic transaminase, aspartate aminotransferase, (GOT) was estimated by the method of Karmen (1955). Aspartic acid and

α-ketoglutaric acid were incubated with serum or urine, $NADH_2$ and malic dehydrogenase.

The rate of formation of NAD was followed at 340 mμ. The unit of GOT is defined as a change in extinction of 0.001 per min.

The reproducibility of the method was investigated by estimating a serum and corresponding urine GOT 5 times each. The coefficient of variation for the serum estimation was 2.3%, for the urine estimation was 8.2% and for the urine:serum ratio was 7.8%. No significant difference in GOT values could be detected between dialysed and untreated urine.

2.4. SELECTIVITY STUDIES USING EXOGENOUS MACROMOLECULES

If selectivity can be assumed to be an estimate of renal permeability in terms of molecular weight, it becomes of great interest to know how far selectivity reflects glomerular permeability and to what extent tubular reabsorption plays a part. These studies using exogenous macromolecules were designed to investigate the relationship between selectivity and glomerular, as opposed to overall renal, permeability.

In renal disease, tubular reabsorption of protein is thought to be non-specific, so that the protein distribution in the glomerular filtrate is the same as that in the urine. This concept was demonstrated by albumin infusion experiments; on changing the serum protein distribution the renal clearance of all the proteins changed accordingly (Hardwicke and Squire, 1955). The protein patterns were studied by filter paper electrophoresis; more precise methods have not been used to study tubular reabsorption. Immunodiffusion was therefore used to study selectivity before, during and after infusion of exogenous albumin, in order to reassess the pattern of tubular reabsorption of protein and the possible effects of reabsorption on selectivity.

An alternative approach to the problem was made by studying the renal permeability of a substance which is not reabsorbed by the tubules. In this case selectivity should be a direct reflection of glomerular permeability. Dextran was chosen, because it is readily available in a form for intravenous injection of suitable molecular weight range, because there are sufficiently sensitive chemical methods of estimation, and because there is almost certainly no tubular reabsorption (Wallenius, 1954).

2.4.1. ALBUMIN

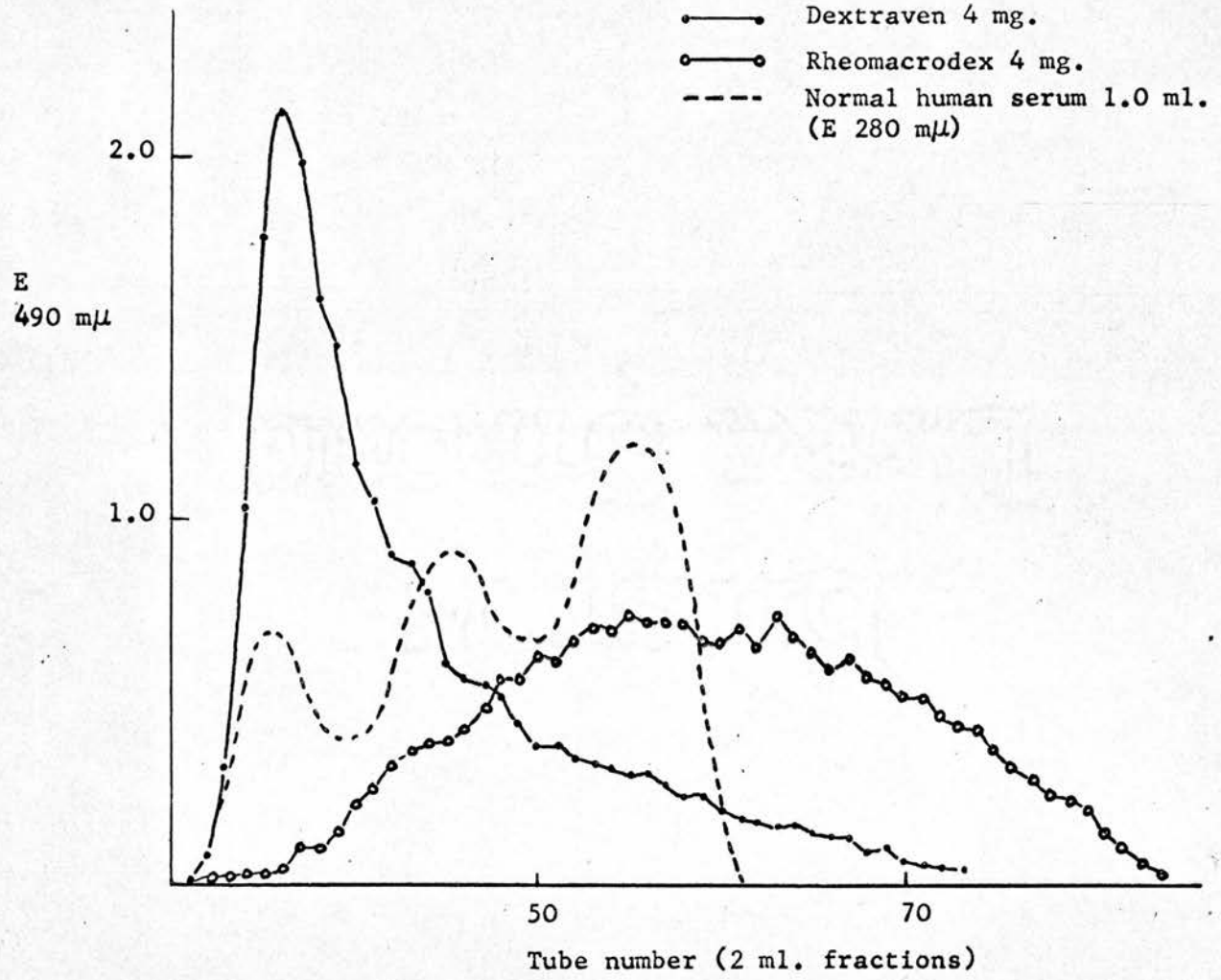
Infusions of albumin were given in some cases of gross proteinuria, generally as part of the therapy, and accompanying selectivity studies were carried out. One case of acute ischaemic renal failure was studied in this way, in view of the doubt about glomerular and tubular handling of protein in this condition.

A control specimen of urine was collected prior to the infusion and a corresponding blood specimen was taken. The patient was then catheterized, the bladder emptied by injection of air and an intravenous infusion of 50 - 100 gm. of albumin commenced. The infusion period lasted between one and two hours. Both during and after the infusion, urine was collected over carefully timed intervals and corresponding blood specimens were taken. The catheter remained in situ during all the collection periods.

Salt-poor human albumin dissolved in sterile, pyrogen free, distilled water was used. Immunoelectrophoresis of the albumin demonstrated only albumin and one faint α_1-globulin arc. Gel filtration however showed the presence of a small proportion of 7S material, which was thought to be due to dimers of albumin; dimerization has been reported to occur in freeze-dried material. (Halwer, Nutting and Brice, 1951; Christiansen, Jenson and Marcker, 1957). An even smaller proportion of 19S material was found, possibly due to formation of trimers. Since the proportion of high molecular weight albumin was small, and since the equilibrium in vivo is unknown, no attempt was made to take this heterogeneity into account.

Selectivity values were estimated from all the urine samples and corresponding sera by immunodiffusion (2.3.1). In addition the total urine protein (2.2.1), total serum protein and concentration of different fractions (2.1) and creatinine clearance (2.1) were estimated.

Fig. 16. Gel filtration of Dextraven and Rheomacrodex



2.4.2. DEXTRAN

Dextran selectivity was studied in selective and unselective patients with various forms of renal disease in order to compare the results with those of the protein studies. In normal subjects and patients with traces of protein in the urine tubular reabsorption of protein is below or only just about the maximum capacity. The contribution to the total urinary protein of degraded protein fragments and proteins from sources other than the serum may be considerable. Dextran studies were therefore of particular interest in this group, in view of the more doubtful interpretation of the protein studies.

Administration of dextran and collection of specimens

Several dextran preparations commercially available were found to be suitable for selectivity studies. A 2:1 mixture of "Dextraven" or "Intradex": "Rheomacrodex" or "Intraflodex" was found to have a molecular weight distribution covering the same range as that of the serum proteins (Fig. 16).

The 2:1 dextran mixture was administered intravenously. In some cases very carefully controlled experiments were carried out. The patient was first catheterized and the bladder was emptied by injection of air. Dextran (22 - 33 g.) was then infused over a 20 min. period and a series of carefully timed urine collections were commenced. Blood specimens were taken at the beginning and end of each collection period, during which time the catheter remained in situ. Four such experiments were carried out in order to study any changes in dextran selectivity over a period of time and to assess the effect on selectivity of changing plasma concentrations of dextran. In the remaining cases the patient was asked to empty his bladder, a single injection of dextran (4 - 11 g.) was given and after 10 - 20 minutes,

the patient was asked to empty his bladder again. In two cases a blood specimen was taken at the mid-point of the collection period. This simple test was in many cases successfully carried out on out-patients.

Estimation of Dextran

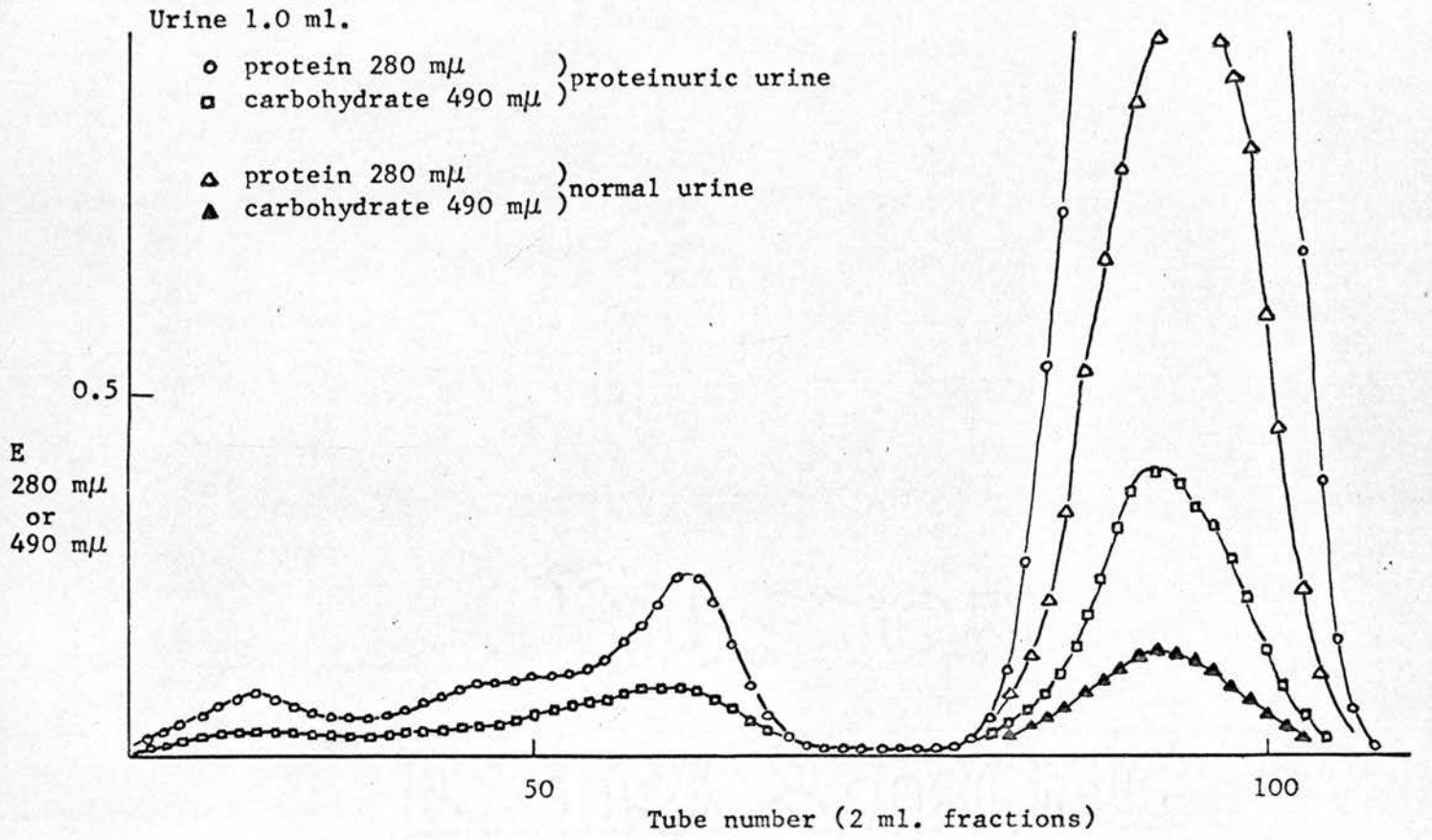
Dextran was estimated by the method of Dubois et al. (1956). This method was chosen in preference to the anthrone method (Semple, 1957) which has a similar sensitivity, because of the indefinite stability of the reagents. Phenol solution, 2 ml. of a 5% w/v, was added to 1 ml. of sample in a boiling tube and 5 ml. of concentrated H_2SO_4 was added to the mixture. In order to ensure rapid and even mixing, the acid was added by means of a "Zippette", the delivery tip being shielded to avoid splashing. The boiling tube was held by tongs while adding the acid. When the tubes were cool the extinction was read in a Unicam SP 600 spectrophotometer at 490 mμ against an appropriate blank, which was prepared by adding the phenol and acid to 1 ml. of distilled water or 1 ml. of the 0.2 M tris saline buffer, pH 8.0, which was used for the column chromatography.

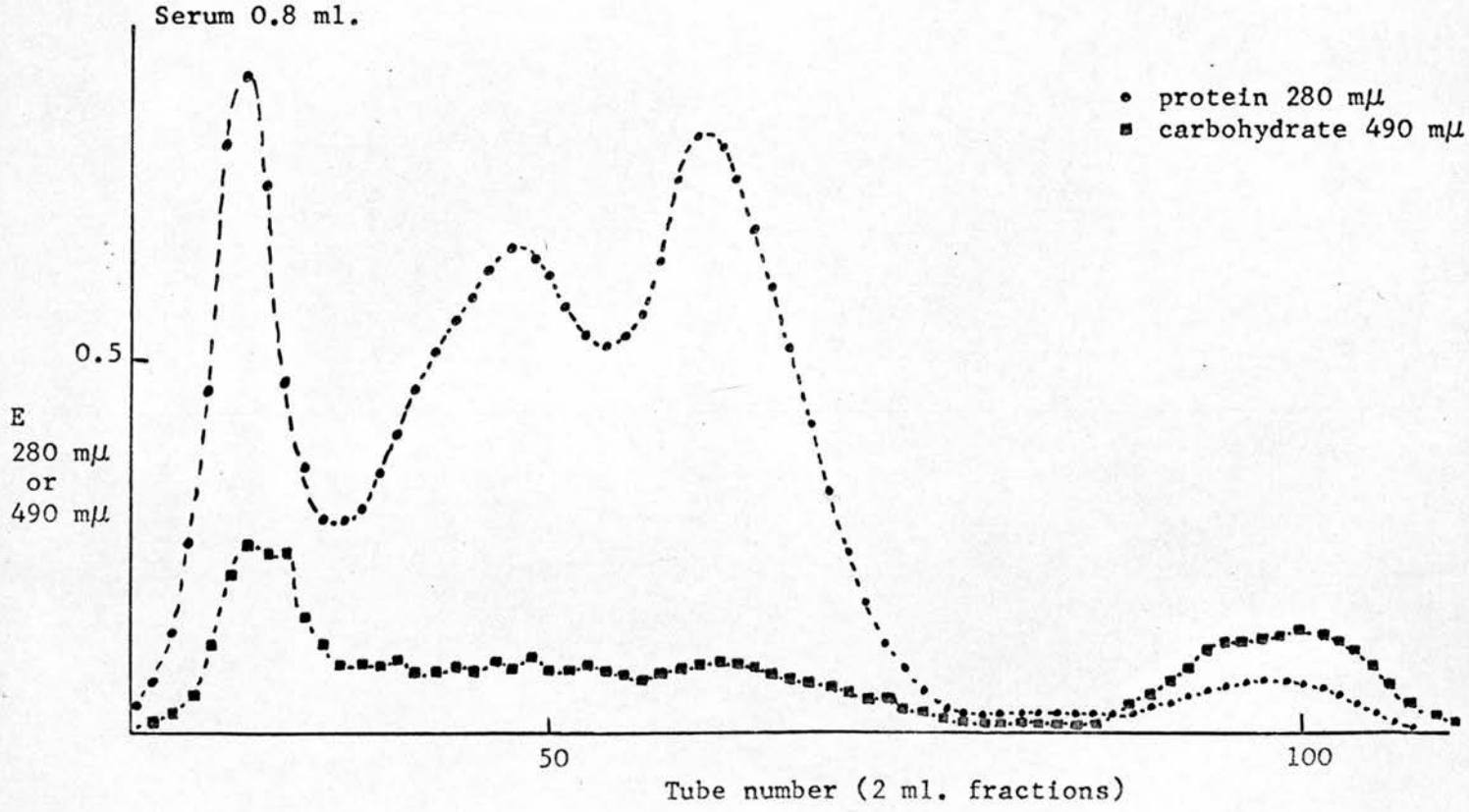
The method was found to be sensitive, reproducible and convenient. Dilutions of Rheomacrodex (10 g.%) were used to make a calibration curve, which was linear over the approximate range 0 - 20 mg.% w/v. Accurate calibration was not essential, since absolute values of dextran were not required. Ten estimations on a single sample gave the mean extinction reading as 0.580, range 0.561 - 0.598, S.D. $\pm$ 0.009 and coefficient of variation 1.5%. The colour developed was stable up to 24 hr. and did not change appreciably over 12 days.

Gel filtration of dextran

Gel filtration, as described earlier (2.2.3), was used to study the molecular weight distribution of the serum and urine dextrans. It has been

Fig. 17. Gel filtration of protein and carbohydrate in urine and serum.

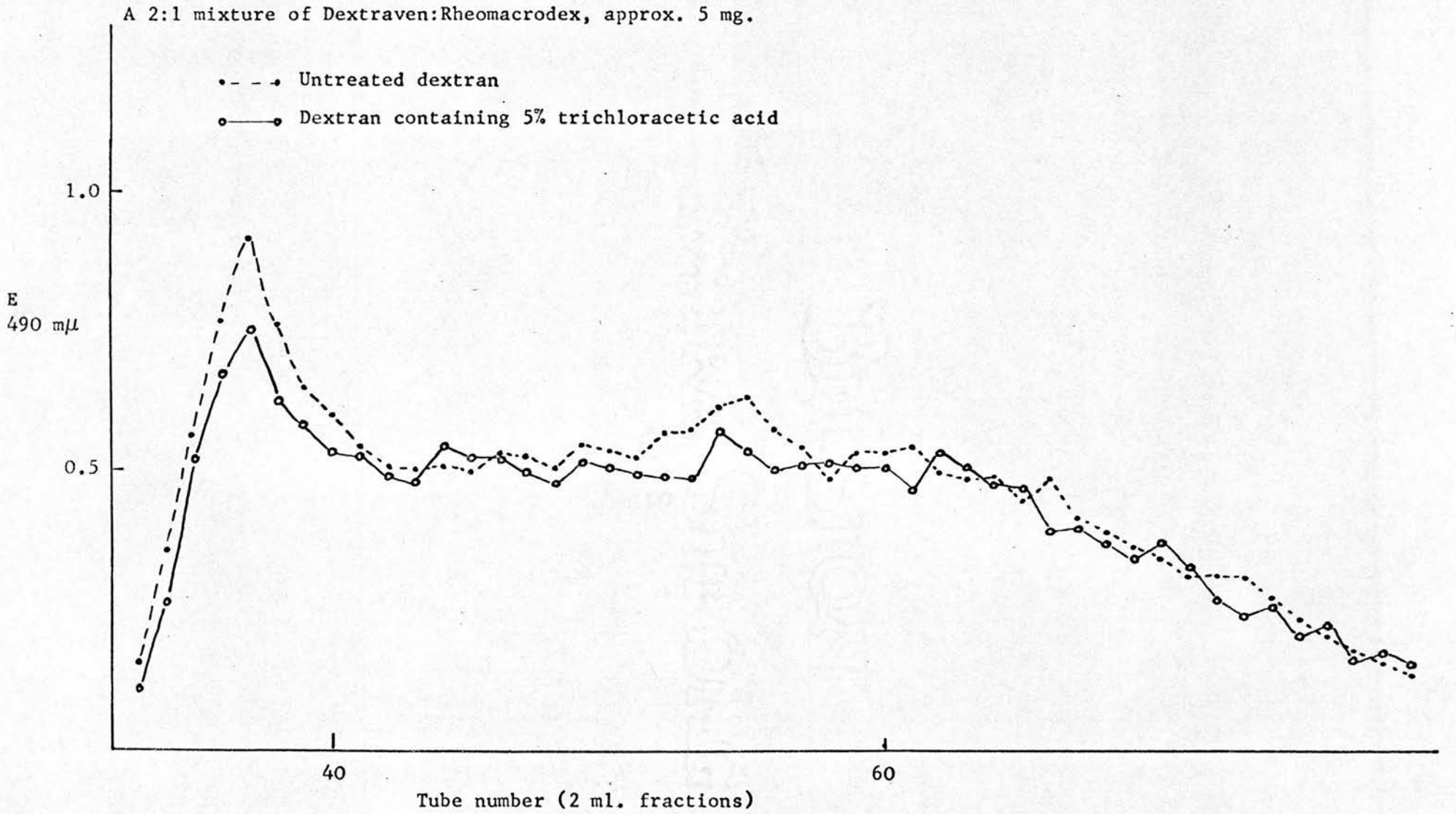




demonstrated that Sephadex separates dextrans, like proteins, on a molecular weight basis (Granath and Flodin, 1961) and presumably the same relationship of elution volume and logarithm of molecular weight applies. In the majority of the single injection experiments a blood specimen was not taken and the urine dextran was compared, by gel filtration, to the injection mixture. The urinary concentration of dextran was first estimated, as described above, generally after appropriate dilution of the sample and protein precipitation by 10% w/v trichloracetic acid, as urine can contain appreciable amounts of protein-bound carbohydrate (Kao et al., 1965). No attempt was made to remove glucose and other sugars, however, so that only an approximate estimation of dextran was obtained. In some cases the urine dextran required to be concentrated before application to the Sephadex column. Concentration was carried out by PEG (2.2.2) and although PEG counter-dialyses it was found to have no effect on the method of estimation of dextran.

The protein-bound carbohydrate present in serum and urine samples (Laurent, 1958) was precipitated by appropriate volumes of 10% or 30% w/v trichloracetic acid, immediately before gel filtration. Fig. 17 shows the amounts of carbohydrate in column effluents from serum and from proteinuric and normal urine samples which contained no dextran. The carbohydrate in serum and proteinuric urine, which eluted over the protein range, is presumably glycoprotein and this was removed by trichloracetic acid. The carbohydrate eluting much later from serum and both normal and proteinuric urine at a single terminal peak was not affected by trichloracetic acid. This component was probably glucose. Normal urine can contain up to 15 mg./100 ml. glucose, and urine from patients with renal disease up to 90 mg./100 ml. (Fine, 1965). A similar peak was eluted on gel filtration

Fig. 18. The effect of trichloracetic acid on dextran.



of the injection mixture. It was therefore necessary to wash the column with at least two elution volumes of buffer between successive runs. Trichloracetic acid had no effect on the dextran injection mixture (Fig. 18) and there was therefore no reason to suppose it would affect the dextran in serum and urine.

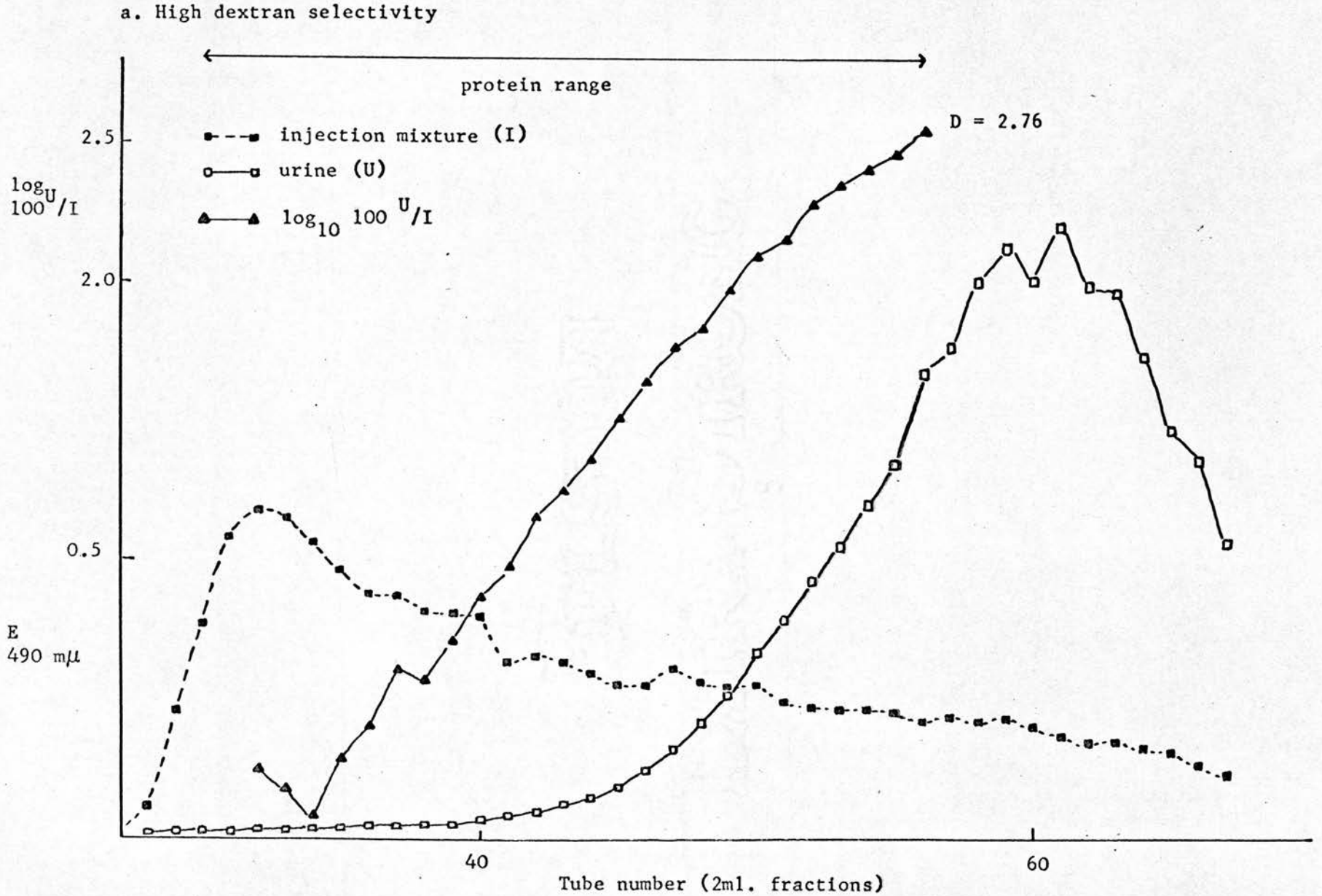
Samples containing 5 - 10 mg. dextran were applied to the Sephadex column. Urine and the corresponding serum or injection mixture were run successively. After every second or third dextran separation a normal serum was run as a marker to check the protein separation pattern of the column. This was necessary as calculations of dextran selectivity were made over the protein range, and the serum or injection mixture had a first peak, but no terminal peak and the urine dextran had only a terminal peak, which was eluted after the third protein peak. The normal serum marker was also essential for lining up the dextran elution patterns, as any change in column separation could not otherwise be detected.

The dextran in the column effluents was estimated. Occasionally a few tubes contained dextran concentrations of over 20 mg.% and the estimation was repeated, using a diluted sample and multiplying the result accordingly. It was found that several effects could give spuriously high dextran values, in particular a faint trace of Sephadex washing from the column into the effluent fractions introduced large errors. In addition, tubes which were not scrupulously clean, tubes which had been dried with acetone, and buffer with any bacterial contamination could also give high readings. Great care was taken to eliminate these effects and a constant check was kept on the column blank and buffer blank.

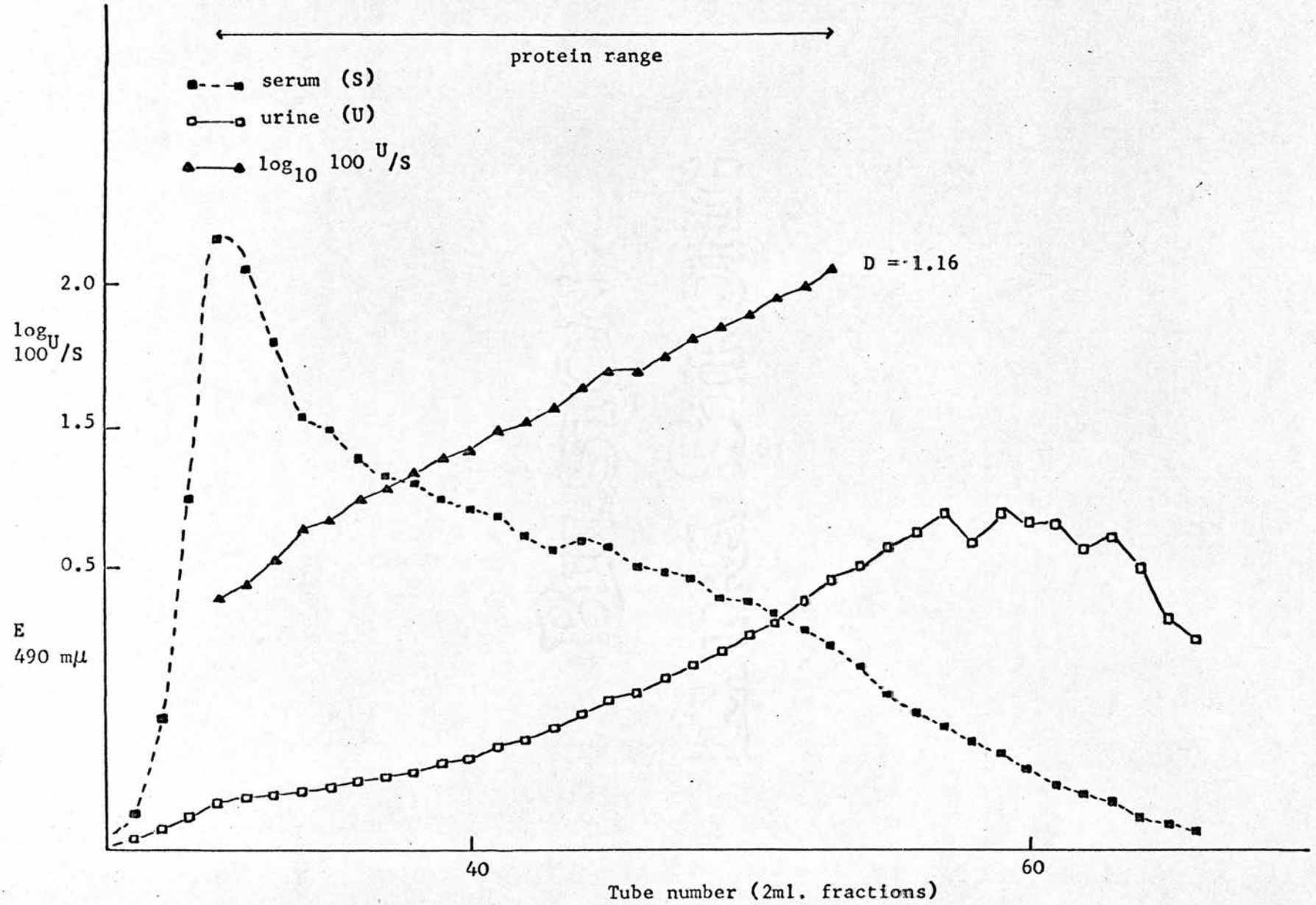
Calculation of dextran selectivity

The dextran index of selectivity was calculated in exactly the same

Fig. 19. Determination of dextran selectivity.



b. Low dextran selectivity



way as the protein index of selectivity (2.3.3). Urine:serum dextran ratios were calculated for tubes between the first and third protein peaks. The logarithm of $\frac{100 \text{ urine dextran concentration (U)}}{\text{serum dextran concentration (S)}}$ was plotted against tube number, when an approximately linear relationship was found. The slope of the regression line was calculated by the method of least squares. The dextran index of selectivity (D) was taken as the total change in $\log_{10} 100\ ^{U}/_{S}$ between the first and third peaks. This index, D, is directly comparable to the protein index, Δ.

Fig. 19 shows typical urine and serum elution patterns and the linear relationship of $\log 100\ ^{U}/_{S}$ to tube number. The molecular weight range of the injected dextran, and hence the excreted dextran, extended to much lower values than that of the serum proteins. It was therefore possible to investigate the relative renal clearance of dextran at lower molecular weights and to compare values with those of the creatinine clearance, providing the urine had not been concentrated prior to gel filtration. In addition to estimating dextran selectivity, total urine protein excretion, creatinine clearance and protein selectivity (both -k and Δ) were determined for every subject.

Errors of the method

The accuracy and possible sources of error were investigated in several ways.

A series of twelve duplicate determinations of D gave a coefficient of variation of 9.1%. This value is slightly higher than that of the protein index, Δ, and is probably due, in part, to the ease with which spuriously high dextran readings are obtained, and in part to the difficulty of aligning urine and serum plots. In addition, application to the column

TABLE 16.

Effect on dextran selectivity of shifting urine elution patterns

Values of selectivity (D) calculated from matching elution patterns compared with values calculated when the urine plot is shifted two tubes to the right or left.

D	2 tubes to R	% error	2 tubes to L	% error
2.67	2.80	+ 4.9	2.72	+ 1.9
2.46	2.29	- 6.9	2.51	+ 2.0
1.84	1.96	+ 6.5	1.71	- 7.1
1.75	1.92	+ 9.7	1.50	-14.0
1.36	1.61	+11.0	1.32	- 2.9

TABLE 17.

The linear relationship between relative clearance and molecular weight of dextran on a log-log plot

The correlation coefficient (r), its limits of significance (p) and the standard error of estimate of log 100 urine/serum (SE) were estimated in 10 subjects with varying values of the index of selectivity (D).

Subject	D	r	p	SE %
1	2.76	0.994	0.001	3.8
2	2.66	0.989	"	7.8
3	2.46	0.985	"	6.1
4	2.10	0.989	"	7.7
5	1.84	0.985	"	5.9
6	1.82	0.954	"	7.3
7	1.75	0.965	"	10.3
8	1.62	0.983	"	4.7
9	1.55	0.992	"	0.9
10	1.12	0.996	"	1.2

TABLE 18.

Effect on dextran selectivity of using an inappropriate serum

Effect of calculating dextran selectivity (D) on basis of serum taken at beginning (A) or at the end(B) of the collection period, or on basis of the injection mixture (I). The proportion of the total dextran given which was lost during the collection period A - B is also shown.

D using mean of A and B	D using A only	% error	D using B only	% error	D using I	% error	% Dextran lost A-B
2.91	2.89	-0.7	2.97	+2.1			7.8
1.82	1.77	-2.7	1.87	+2.7	1.77	-2.7	7.6
1.75	1.77	+1.1	1.71	-2.3			1.1
1.12	1.10	-1.8	1.14	+1.8	0.97	-13.0	4.7
2.46					2.50	+1.6	11.9

was complicated by the fact that the samples were virtually colourless and it was difficult to tell if an even layer was being applied. Because of this high coefficient of variation most of the dextran indices of selectivity were estimated in duplicate.

The importance of aligning urine and serum plots was discussed earlier (2.2.3). Apart from the serum marker runs, there was no way of indicating whether or not the dextran elution patterns were correctly aligned. The errors involved when plots were deliberately shifted are shown in Table 16. A shift of two tubes to the right introduces a mean error of + 8.7%, and one to the left a mean error of - 4.0%. These values are less than those for protein shifts, probably because the dextran elution patterns do not have such rapid changes in slope.

The apparent linear relationship of $\log_{10} 100\ U/S$ and tube number was found in 10 experiments to have highly significant correlation coefficients ranging from 0.954 - 0.996 with a mean of 0.983. Table 17 shows the results, together with the standard error of estimate of the values of $\log_{10} 100\ U/S$.

The carefully controlled experiments enabled a comparison to be made of the values of D which were calculated from serum taken at the beginning, middle and end of each collection period. The results are shown in Table 18. Table 18 also shows the effect of calculating D on the basis of the injection mixture as opposed to a serum taken at the mid point of the first collection period. It can be seen that in spite of a large dextran loss into the urine, the errors in D, when calculated on the basis of an inappropriate serum, are not appreciable.

There is no change in molecular composition of dextran after five years when stored at $4^{\circ}C$ (Maycock and Ricketts, 1961). However, a dextran

TABLE 19.

Stability of dextran

Indices of dextran selectivity (D) were determined before and after storage at 4°C for varying time intervals. The pH of the urine is also shown.

Initial D	Time stored	Repeat D	% error	pH of urine
1.77	6 weeks	1.76	0.6	8.7
1.10	8 weeks	1.27	15	7.8
2.97	16 weeks	very little urinary dextran found		7.1

splitting enzyme has been found in animal tissues, including the kidney (Rosenfeld and Lukomskaya, 1957). The stability of dextran in serum and urine was tested by repeating selectivity estimations after storing at $4^{o}C$ for varying intervals. The results are shown in Table 19. The error becomes appreciable after 2 months and at 4 months very little urinary dextran could be detected. The dextran splitting enzyme described by Rosenfeld and Lukomystaya (1957) has a pH optimum of 4.8, although some preparations have activity at pH 7.0. It is interesting to note that the errors in selectivity are also related to the pH of the urine. Because of the uncertain stability of dextran in urine the dextran selectivity was always determined within 2 weeks of collecting the specimens.

The estimation of dextran selectivity was a less accurate and more time consuming procedure than the estimation of protein selectivity either by immunodiffusion or gel filtration. However, the method was practicable, the techniques involved were simple, and it was possible to study a number of patients and normal subjects in this way.

3. RESULTS

3.1 Values of selectivity in renal disease

3.1.1 Indices of protein selectivity

3.1.2 Clearances of other individual proteins and enzymes

3.1.3 Dextran studies

3.2 Studies on protein selectivity in renal disease

3.2.1 Variation of selectivity values

3.2.2 Selectivity and steroid treatment

3.2.3 Selectivity in relation to other parameters

3.2.4 Selectivity in relation to diagnosis

3.2.5 Selectivity and glomerular ultrastructure

3.3 Values of selectivity in normal subjects

The results are considered in three sections; data from the studies on patients with renal disease are given in Sections 1 and 2 and results from the studies on normal subjects are given in Section 3.

In Section 1 the ranges of values of protein selectivity, obtained using different methods, are given first. These results are compared, and, since there is a good correlation between the methods, the principal method - the immunodiffusion method - is subsequently used for all the studies presented in Section 2. In Section 1 results of clearances of enzymes and of other individual proteins and of the dextran studies are also given.

Section 2 deals with results obtained by studying protein selectivity in renal disease in relation to various factors, which include the specific diagnosis, biological variation, response to steroid treatment, etc. Selectivity is also considered in relation to the ultrastructural glomerular changes.

In Section 3 the indices of selectivity in normal subjects, obtained by different protein methods, and by the use of dextran, are presented and compared.

3.1. VALUES OF SELECTIVITY IN RENAL DISEASE

Selectivity studies were carried out on 130 patients. All had an abnormal amount of urinary protein, as defined by an albumin serum:urine ratio of less than 400, or a total protein excretion of over 200 mg./24 hr. The majority, 99 patients, had a total urinary protein excretion of over 1 gm./24 hr. Of the 31 who had under 1 gm. urinary protein/24 hr., 15 were patients with postural proteinuria and acute ischaemic renal failure, and the remainder included patients with mild proliferative glomerulonephritis, lupus nephritis, multiple myeloma, exercise haematuria and proteinuria of unknown origin.

Renal biopsies were obtained from 119 of the patients and a specific diagnosis was made in all cases. Of the 11 patients who did not have a renal biopsy, all had clinical features which were characteristic of certain types of renal disease, and a definite diagnosis was therefore possible in the absence of histological evidence. Patients in this group had either lupus nephritis, diabetic glomerulosclerosis, amyloid disease, potassium depletion with hypertension, chronic renal failure or acute ischaemic renal failure.

The different types of renal disease that were studied and the number of patients in each group were as follows:

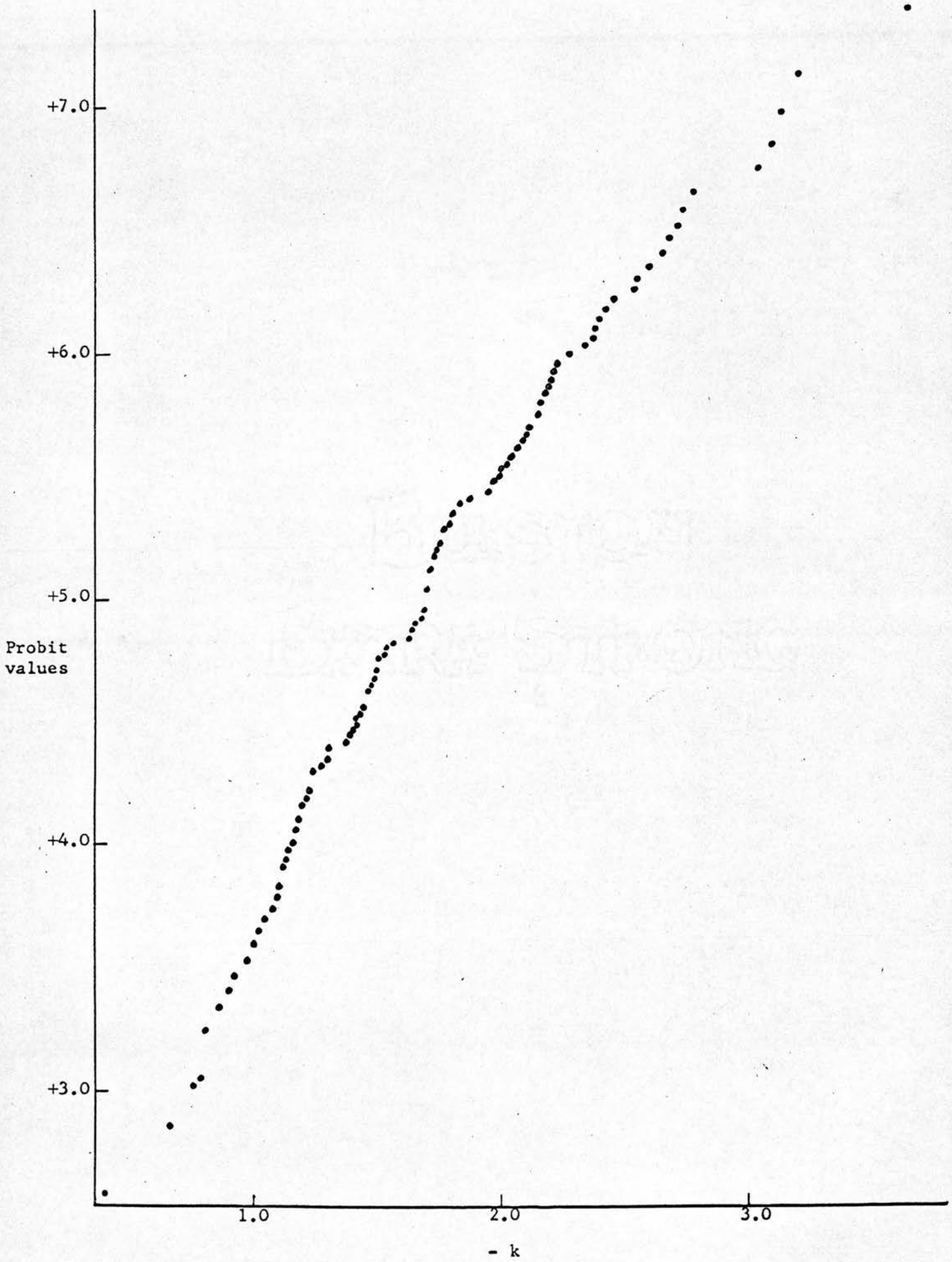
Membranous glomerulonephritis	11
Minimal lesion "	12
Proliferative "	37
Mixed membranous and proliferative glomerulonephritis	5
Lupus nephritis	10
Diabetic glomerulosclerosis	9
Amyloid disease	7

Chronic renal failure	7
Acute ischaemic renal failure	10
Postural proteinuria	5
Miscellaneous	17

The miscellaneous group included patients with potassium depletion and hypertension, renal vein thrombosis, exercise haematuria, pyelonephritis, multiple myeloma and toxaemia of pregnancy.

In addition a preliminary study of proteinuria during toxaemia and accidental haemorrhage of pregnancy was carried out on 14 patients, none of whom had a renal biopsy.

Fig. 20. Indices of protein selectivity by immunodiffusion on a probit scale.



3.1.1. INDICES OF PROTEIN SELECTIVITY

Immunodiffusion

Every patient was studied by this method. Indices of selectivity were estimated for many patients at regular intervals, particularly in cases where there was a change in renal function or in the level of urinary protein excretion. A minimum of three determinations was carried out on 84% of the patients studied; a single estimation of selectivity was made in only 10% of the patients.

Values for the index of selectivity ranged from 0.40 - 3.82. Since selectivity values were, in general, found not to vary significantly over long intervals of time (3.2.1), and were not affected when steroid treatment was commenced while proteinuria persisted (3.2.2), a mean value for each patient was taken. The mean index of selectivity for the whole group of patients was 1.74, S.D. $\pm$ 0.61, coefficient of variation 35%, standard error of the mean 3.1%. The indices of selectivity were plotted on a probit scale (Fig. 20), when the distribution of selectivity values was seen to be consistent with a normal, bell-shaped distribution, as indicated by the linearity of the plot.

Gel filtration

Selectivity values were estimated on 51 patients by gel filtration. The patients suffered from similar types of renal disease to those of the whole series of patients studied.

Determinations were carried out 61 times on 44 patients with proteinuria of over 1 gm./day. Values for the index of selectivity ranged from 0.40 - 2.51. Taking a mean value for each patient, the mean for the group was 1.36, S.D. $\pm$ 0.48, coefficient of variation 35%, standard error of the mean 7.2%. The indices of selectivity were plotted on a probit scale (Fig. 21),

Fig. 21. Indices of protein selectivity by gel filtration on a probit scale.

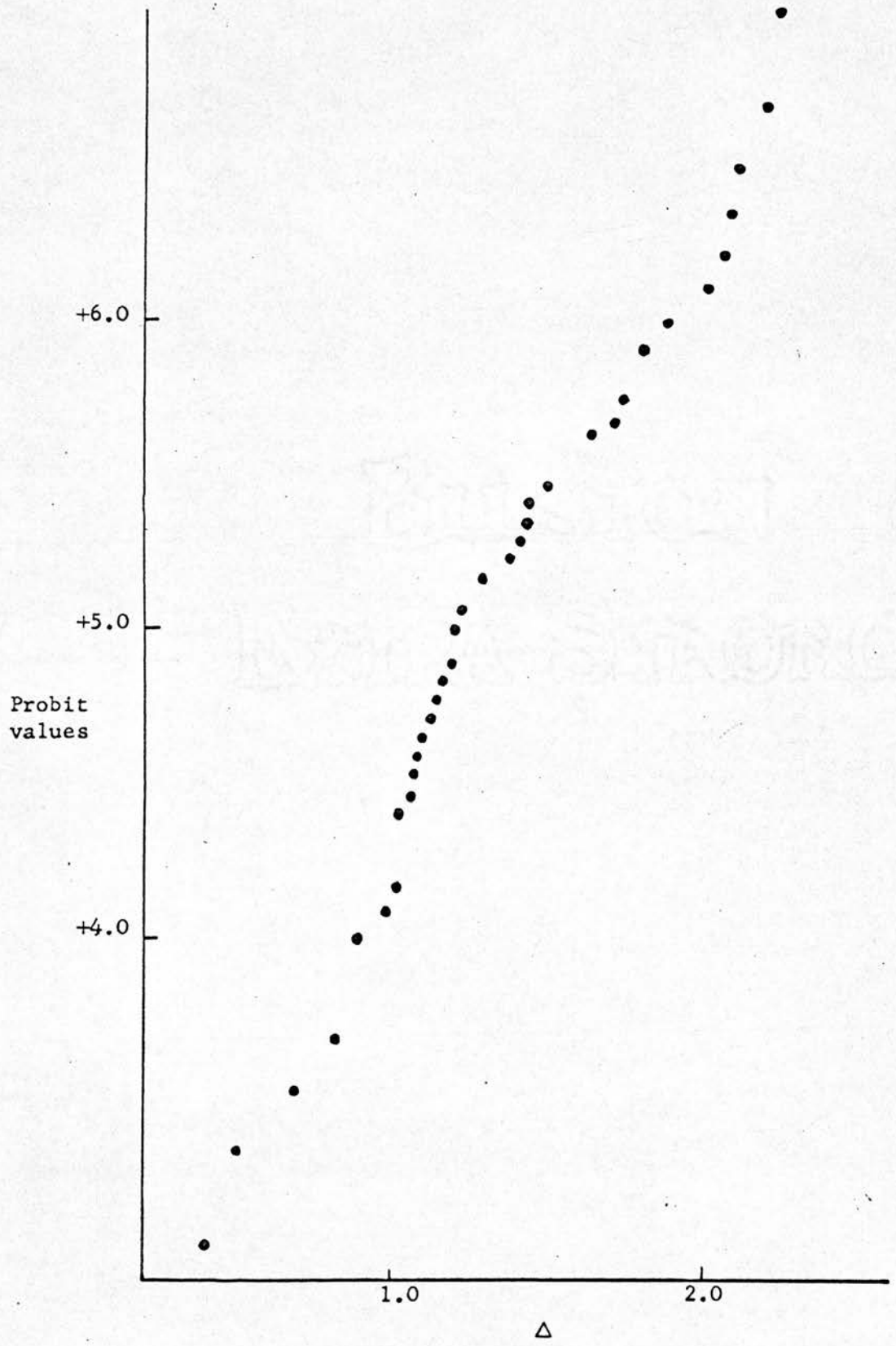
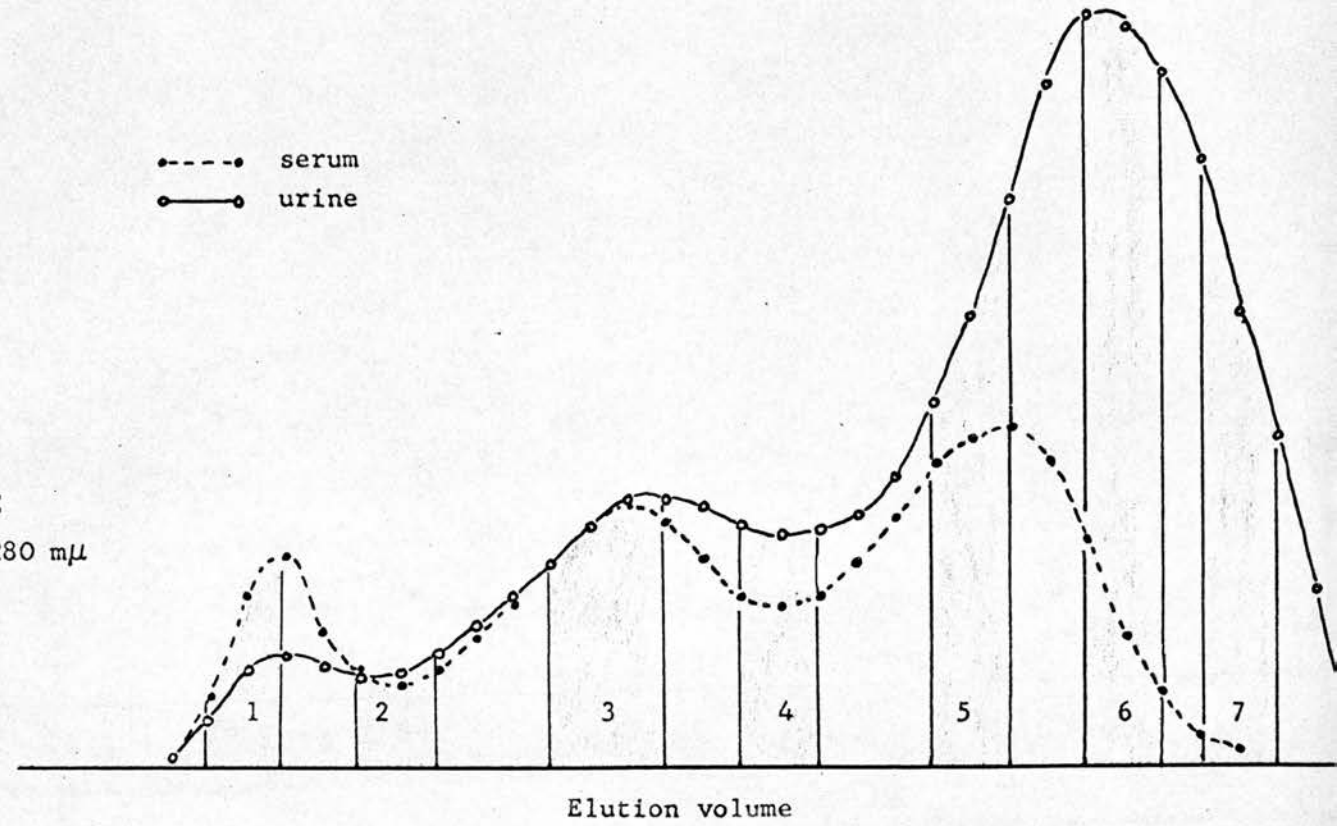


Fig. 22. Immunoelectrophoretic examination of serum and urine proteins after gel filtration.



The shaded areas represent fractions which were examined by immunoelectrophoresis.

o =present in urine, + = present in serum.

	1	2	3	4	5	6	7
γ_{1M}-globulin	+o						
α_2-β-lipoproteins	+o	+o					
α_2-macroglobulin	+o	+o					
haptoglobin	+o	+o	+o	+o	+		
high MW α_1-globulin		+o	+o				
β_{1A}/β_{1C}-globulin		+	+o				
ceruloplasmin		(+o)	+o	o			
γ_{1A}-globulin		+	+o	o	o		
γ-globulin		+o	+o	+o			
α_2-globulin			+o	+o			
albumin			(+o)	+o	+o	+o	o
hemopexin				+o	+o	+o	o
transferrin				+o	+o	+o	o
α_1-glycoprotein				+o	+o	+o	o
orosomucoid				+o	+o	+o	o

when the distribution of selectivity values was seen to be consistent with a normal, bell-shaped distribution, as indicated by the linearity of the plot.

Determinations were carried out 11 times on 8 patients with proteinuria of under 1 gm./day. In this group the correlation coefficient of the log-log plot of renal clearance against molecular weight of protein was less significant (2.3.2) and values of selectivity were much lower than in patients with proteinuria of over 1 gm./day. The mean index of selectivity for the group was 0.89, range 0.47 - 1.60. In one patient with minimal lesion glomerulonephritis, selectivity values were estimated both when proteinuria was over 1 gm./day and when proteinuria was under 1 gm./day. Although the value obtained by immunodiffusion was consistent, the value obtained by gel filtration fell significantly when the proteinuria diminished.

Immunoelectrophoretic analysis of gel filtration elution patterns

Serum and urine protein fractions obtained by gel filtration were examined immunoelectrophoretically. Serum proteins from normal subjects and from three patients with the nephrotic syndrome, and urinary proteins from three patients with the nephrotic syndrome were investigated in this way. The results were similar and a typical experiment is shown in Fig. 22.

In both serum and urine the proteins were eluted according to their molecular weights (given in Table 4). All the identifiable proteins seen in the serum fractions were detected in the corresponding urine fractions. In addition to confirming the separation on G 200, these experiments also enabled an approximate molecular weight to be estimated for those proteins where this value was not known. The approximate values were useful in interpreting selectivity patterns determined by immunoelectrophoresis (see below). A high molecular weight α_1-globulin was found to elute with the 11-7S material.

TABLE 20.

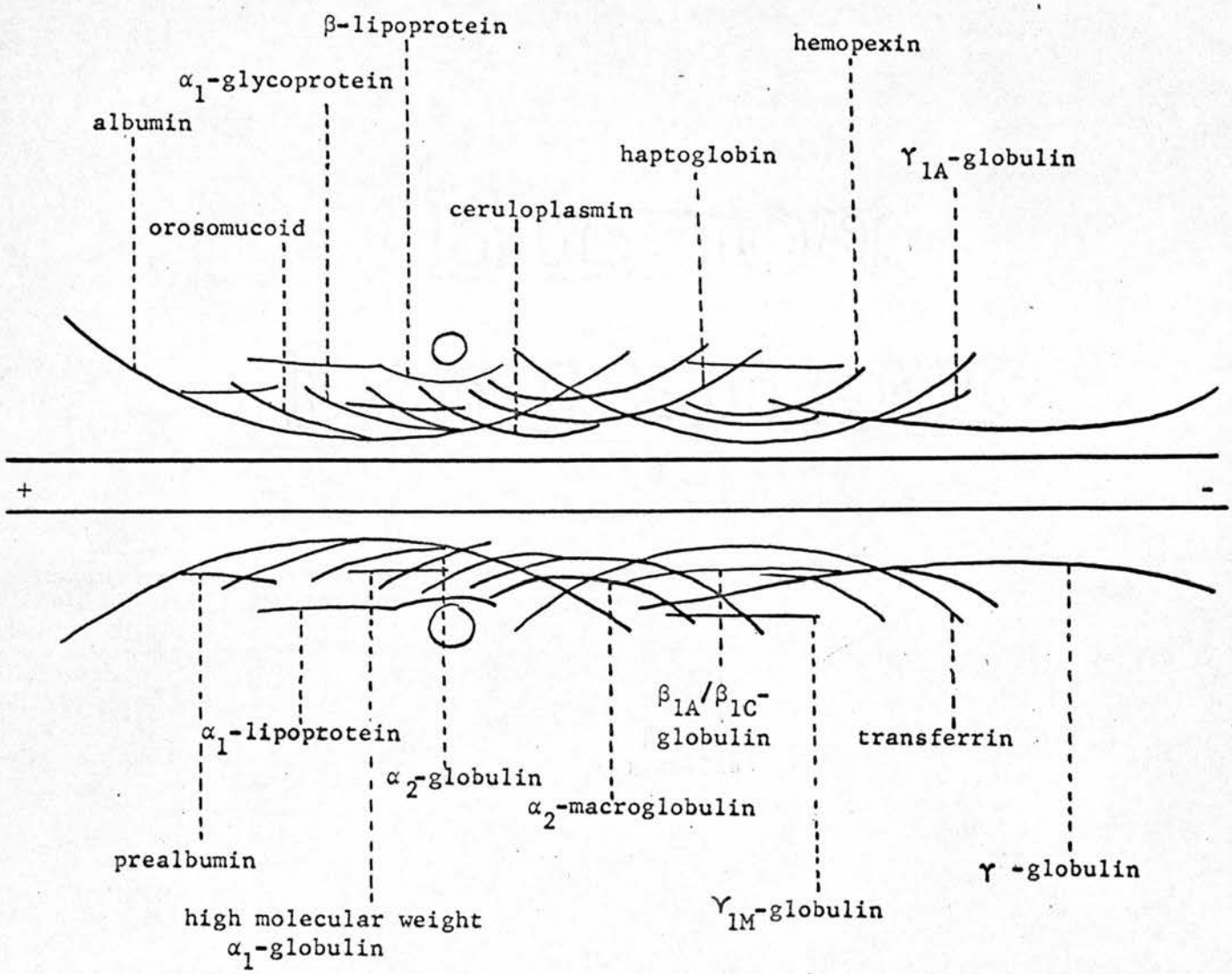
Components seen on immunoelectrophoretic examination of urine proteins

The following proteins were identified in some or all of the urines: orosomucoid (O), albumin (A), transferrin (S), Υ-globulin (Υ), α_1-glycoprotein (α_1G), hemopexin (He), haptoglobin (Ha), ceruloplasmin (C), β_{1M}/β_{1C}-globulin (βAC), Υ_{1A}-globulin (Υ_{1A}), high molecular weight α_1-globulin (α_1H), α_2-macroglobulin (α_2M), β-lipoprotein (βL), Υ_1-macroglobulin (Υ_{1M}), prealbumin (p). Additional unidentified proteins (Add) and the total number of components are also shown.

Subject	O	A	S	Υ	α_1G	He	Ha	C	βAC	Υ_{1A}	α_1H	α_2M	βL	Υ_{1M}	p	Add	Total
Group HS 1	+	+	+	(+)													4
2	+	+	+	(+)		+											5
3	+	+	+	+		+											5
4	+	+	+	+	+											1	6
5	+	+	+	(+)	+	(+)	(+)										7
Group S 6	+	+	+	+	+					+	+						7
7	+	+	+	+	+		+	+									7
8	+	+	+	(+)	+	+		+									8
9	+	+	+	+	+	+	+	+									8
10	+	+	+	+	+	+	+	+								$2\alpha_2$	10
11	+	+	+	+	+	+	+	+	+								8
12	+	+	+	+	+		(+)	+		+							8
13	+	+	+	+	+		+	+	+								8
14	+	+	+	+	+	(+)	+	+	+								9
15	+	+	+	+	+	+	+	+	+								9
16	+	+	+	+	+	+	+	+	+								9
17	+	+	+	+	+	+	(+)	+	+							1β	10
18	+	+	+	+	+	+	+	+	+	+							10
19	+	+	+	+	+	+	+	+	+	+							10

	20	+	+	+	+	+	+	(+)	+	+	+						$1\alpha_1$	11
	21	+	+	+	+	+	+	(+)		+		+						10
	22	+	+	+	+	+	+	(+)	+	+			(+)					10
Group I	23	+	+	+	+	+	+	+		+	+	+						10
	24	+	+	+	+	+	+	+	+	+			+					10
	25	+	+	+	+	+	+	+	+	+		+					1β	11
	26	+	+	+	+	+	+	+	+	+	+	+						11
	27	+	+	+	+	+	+	+	+	+	+		+				1β	12
	28	+	+	+	+	+	+	+	+	+	+		+				1β	12
	29	+	+	+	+	+	+	+		+	+		+				$1\alpha_1, 1\alpha_2$	12
	30	+	+	+	+	+	+	+	+	+	+	+	+			+		13
	31	+	+	+	+	+	+	+	+	+	+	+	(+)				2β	14
Group U	32	+	+	+	+	+	+	+	+	+	+	+	+		+			13
	33	+	+	+	+	+	+	+	+	+	+	+	+	+				13
	34	+	+	+	+	+	+	+	+	+	+	+	+	(+)				13
	35	+	+	+	+	+	+	+	+	+	+	+	+	+			1	14
	36	+	+	+	+	+	+	+	+	+	+	+	+	+			$1\alpha_2$	14
	37	+	+	+	+	+	+	+	+	+	+	+	+	+			1β	14
	38	+	+	+	+	+	+	+	+	+	+	+	+	+		+	$1\alpha_1$	15
	39	+	+	+	+	+	+	+	+	+	+	+	+	+		+	$1\alpha_1$	15
	40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	$2\beta, 1\alpha_2$	18

Fig. 23. Immunoelectrophoresis of normal human serum.



Haptoglobin was found to elute over a wide range of elution volume, but it is known to exist in more than one molecular form. β_{1A}/β_{1C}-Globulin eluted with the 7S material and hemopexin with the 4S material. An unidentified α_2-globulin was found to elute with the 7S material.

Immunoelectrophoresis

Forty patients were investigated by immunoelectrophoresis. The different types of renal disease studied were similar to those of the whole series of patients studied.

The maximum number of precipitin arcs that could be identified in pooled normal human serum was 17 (Fig. 23). All these components could usually be seen in the patients' serum, although some of the smaller proteins, such as albumin, transferrin and γ-globulin were sometimes reduced, and some of the larger proteins such as α_2-macroglobulin and β-lipoprotein were increased. However, since the technique is only a qualitative one, only gross serum changes produced any significant change on immunoelectrophoresis.

The urines examined contained between 4 and 18 components. Albumin, transferrin, γ-globulin, and orosomucoid were always seen. The frequency with which other proteins were detected varied according to their molecular weights (given in Table 4). An estimate of the molecular weights of some proteins which were not known was obtained from gel filtration experiments (Fig. 22).

Details of the proteins detected in all the urines are shown in Table 20. Occasionally, when a protein was not seen in the urine it could not be detected in the serum, but this was only true of hemopexin, ceruloplasmin, the high molecular weight α_1-globulin, γ_{1M}-globulin and prealbumin. The other proteins were always detected in the serum. Selectivity was judged on the number and molecular weights of the components seen in the urine, in

TABLE 21.

Criteria of determination of selectivity of proteinuria by immunoelectrophoresis

Selectivity	Proteins always detected in urine	Proteins sometimes detected in urine	Total no. of components in urine
Highly selective Group HS	Albumin Transferrin γ-Globulin Orosomucoid	α_1-Glycoprotein Hemopexin Haptoglobin	4 - 7
Selective Group S	Albumin Transferrin γ-Globulin α_1-Glycoprotein orosomucoid	Hemopexin Haptoglobin Ceruloplasmin β_{1A}/β_{1C}-Globulin γ_{1A}-Globulin	7 - 11
Intermediate Group I	Albumin Transferrin γ-Globulin α_1-Glycoprotein Hemopexin Haptoglobin β_{1A}/β_{1C}-Globulin orosomucoid	Ceruloplasmin High M.W. α_1-globulin α_2-Macroglobulin	11 - 14
Unselective Group U	Albumin Transferrin γ-Globulin α_1-Glycoprotein Hemopexin Haptoglobin β_{1A}/β_{1C}-Globulin γ_{1A}-Globulin High M.W.α_1-globulin α_2-Macroglobulin orosomucoid	β-Lipoprotein γ_{1M}-Globulin	13 - 18

Fig. 24. Determination of selectivity of proteinuria by immunoelectrophoresis.

Serum (S) and urine (U) patterns from patients with different degrees of selectivity. Selectivity was judged according to the criteria given in Table 21.

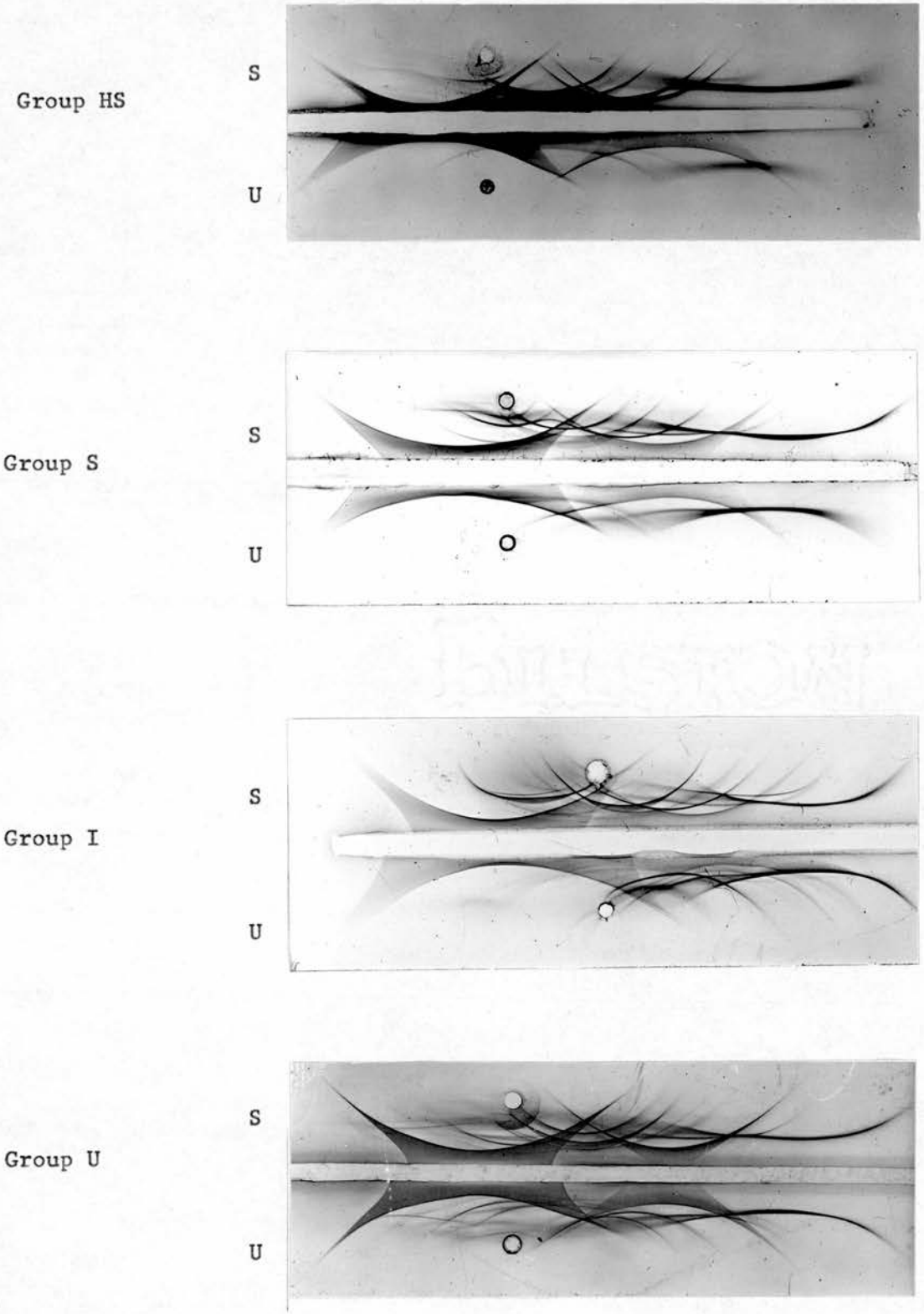
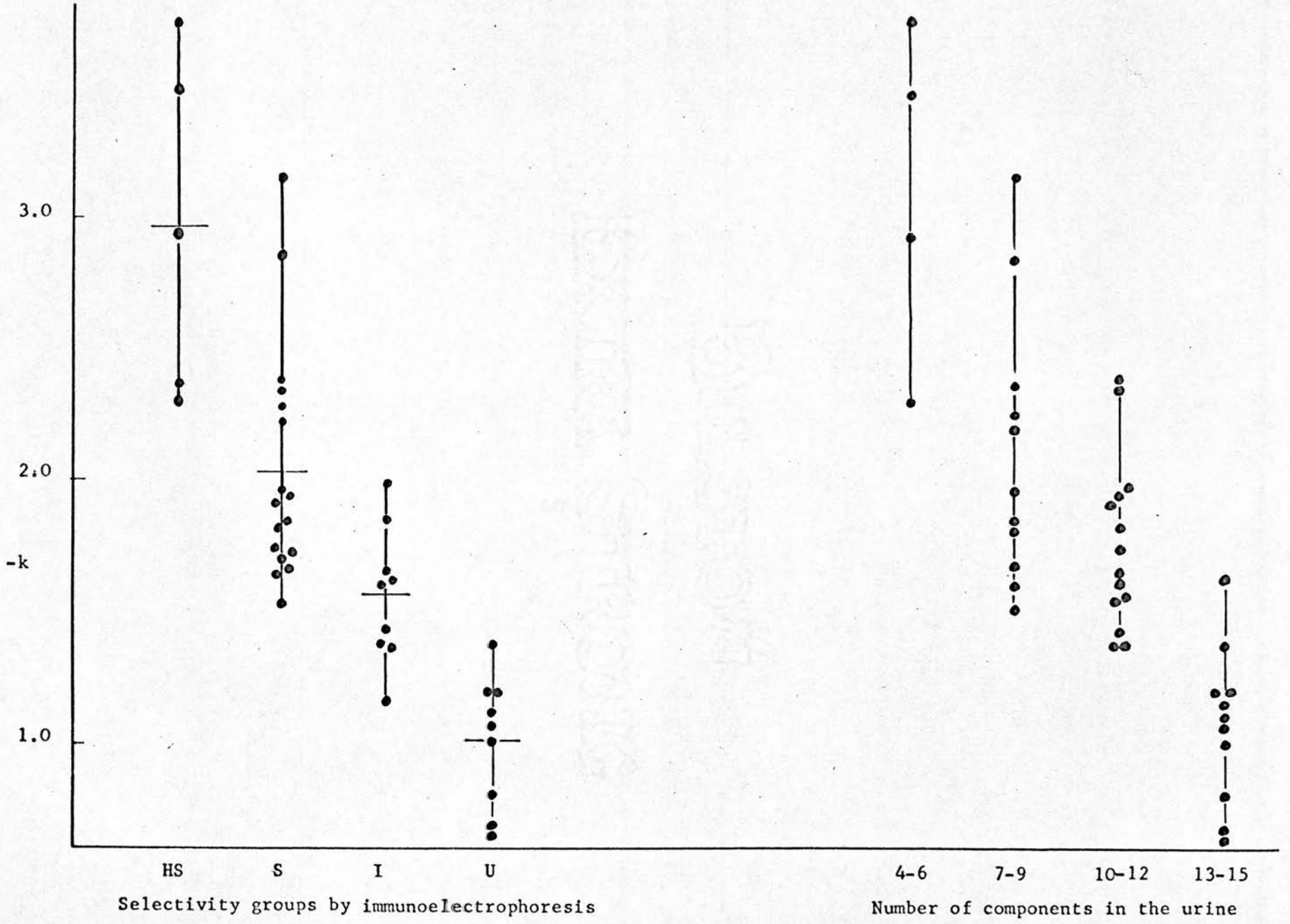


Fig. 25. Correlation of values of selectivity determined by immunodiffusion and immunoelectrophoresis.



relation to the serum. Four groups of patients were distinguished on this basis. The criteria are given in Table 21. Of the 40 patients, 5 were highly selective (HS), 19 were selective (S), 9 were intermediate (I), and 9 were unselective (U).

Additional unidentified α- and β-globulins were sometimes seen in the urine, particularly in groups I and U. A split γ-globulin arc, indicating the presence of a low molecular weight, possibly degraded, molecule, was only seen in two urines (Patients 4 and 35). Prealbumin was only detected in a few urines in groups I and U.

Fig. 24 shows stained immunoelectrophoretic patterns of serum and urine from a patient in each selectivity group.

Correlation of protein selectivity methods

Selectivity values obtained by immunodiffusion and immunoelectrophoresis were compared in 40 patients. Fig. 25 shows the selectivity groups distinguished by immunoelectrophoresis, plotted against the corresponding indices of selectivity obtained by immunodiffusion. There was a striking correlation. Mean values of selectivity derived from immunodiffusion studies (-k), for the four immunoelectrophoretic groups, were 2.96 for group HS, 2.03 for group S, 1.57 for group I, and 1.01 for group U; corresponding ranges of values were 3.74 - 2.30, 3.16 - 1.53, 1.99 - 1.16, 1.38 - 0.64. The range of -k for adjacent groups therefore overlapped, but there was no overlap of -k values between the highly selective and intermediate groups, or between the selective and unselective groups.

Fig. 25 also shows a comparison between -k values and the number of components seen in the urine. The correlation was not so striking, and only the extreme groups with 4 - 6 and 12 - 15 components had no overlap in immunodiffusion selectivity values.

Fig. 26. Correlation of individual values of selectivity determined by immunodiffusion and gel filtration.

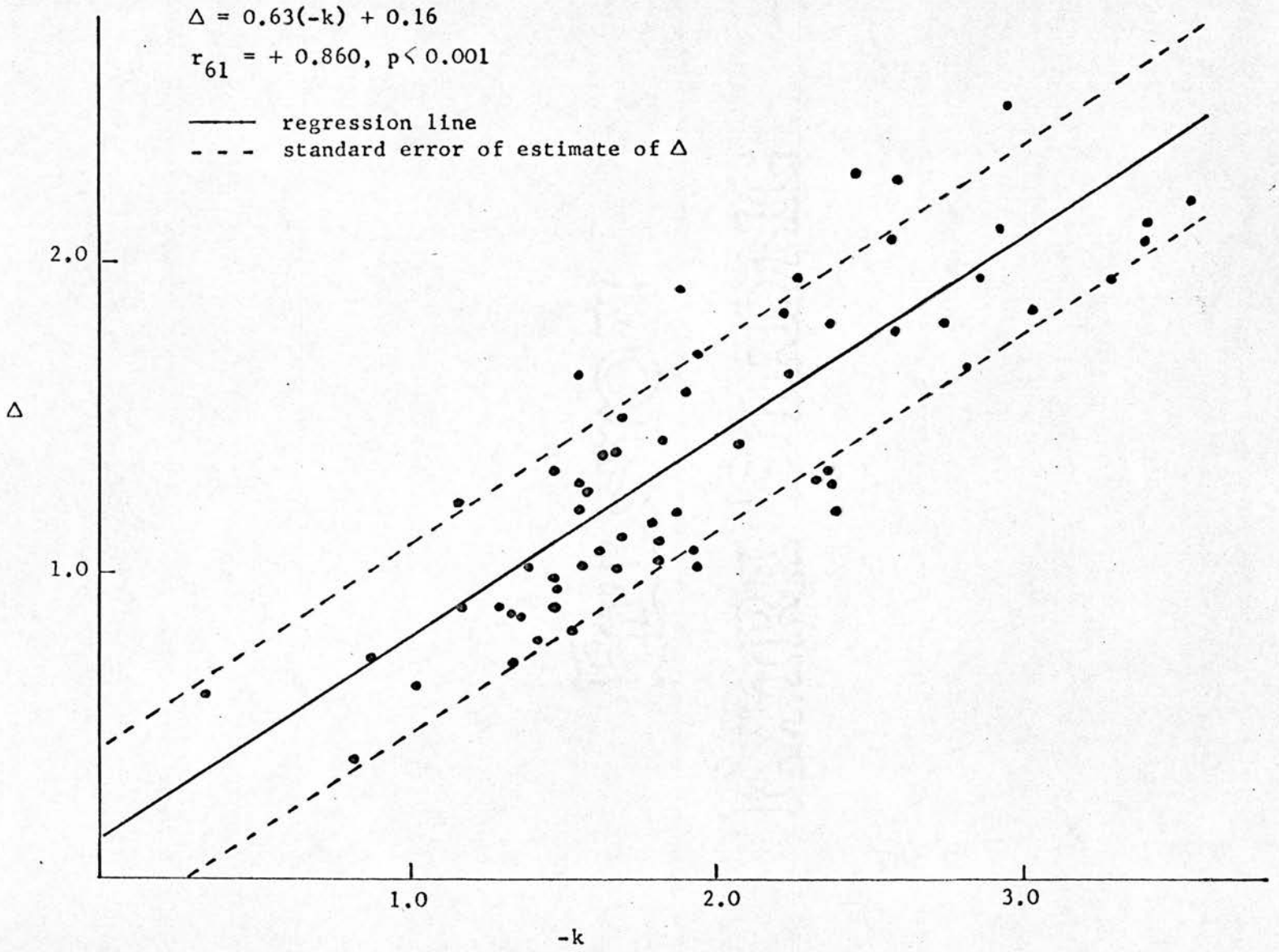


Fig. 27. Correlation of mean values of selectivity determined by immunodiffusion and gel filtration.

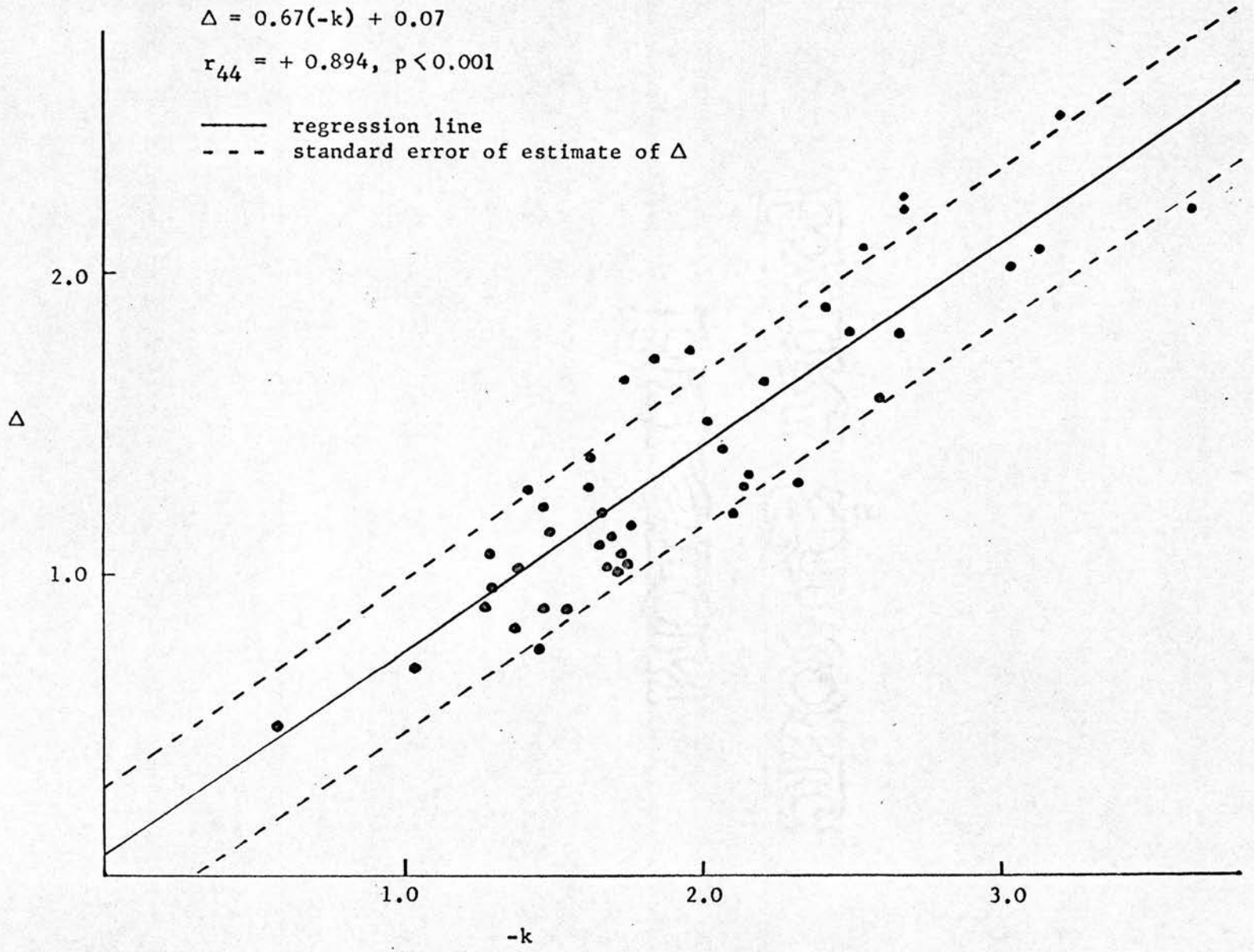
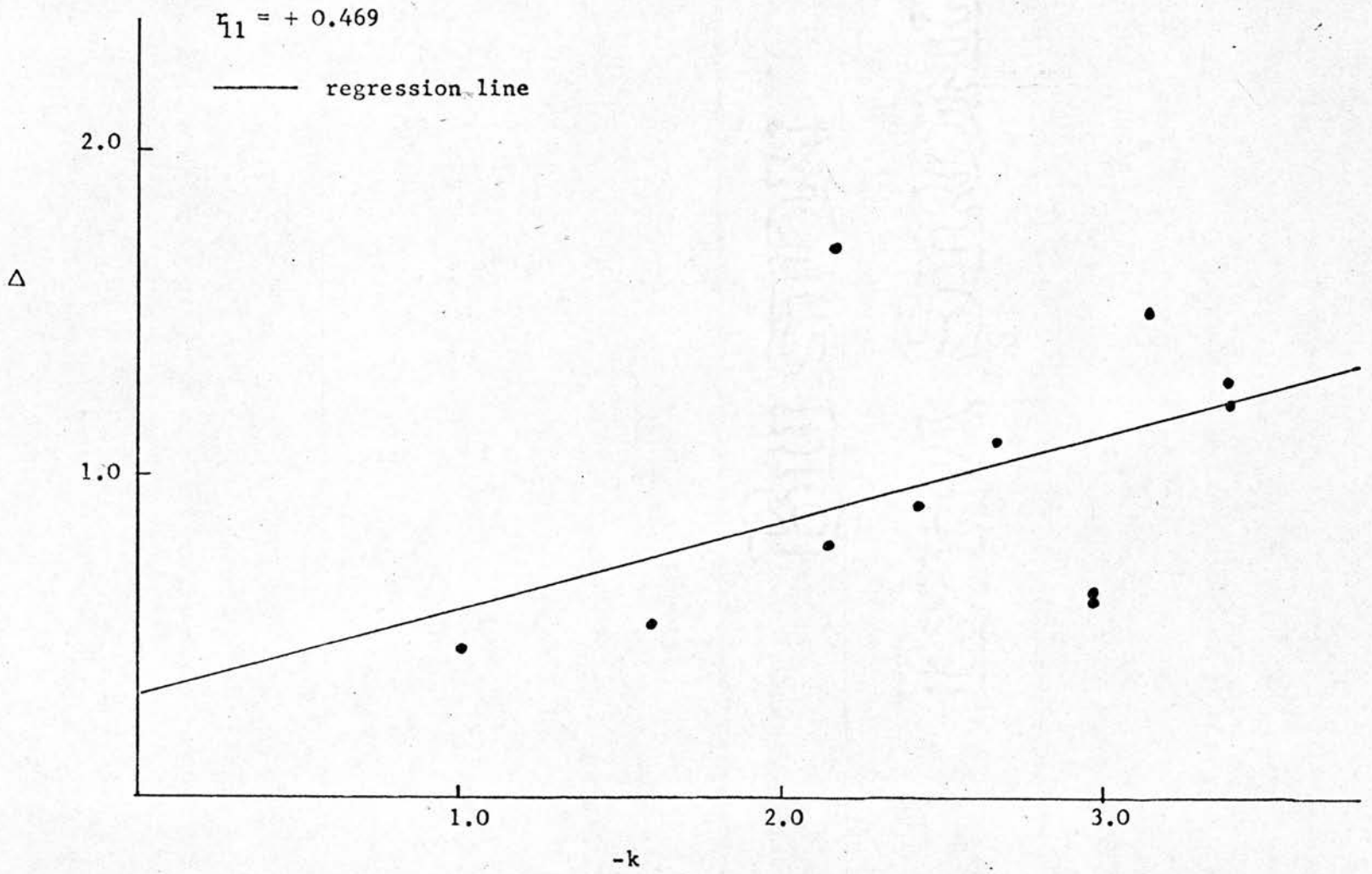


Fig. 28. Correlation of values of selectivity determined by immunodiffusion and gel filtration in proteinuria of under 1g./24 hr.



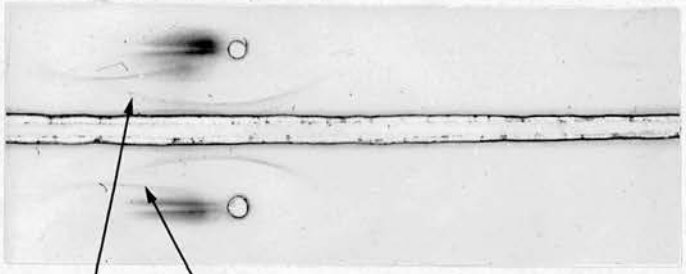
Indices of selectivity estimated on 72 specimens by gel filtration (Δ) and immunodiffusion (-k) were compared. Sixty-one specimens were obtained from patients with proteinuria of over 1 g./day, and Fig. 26 shows the 61 values of Δ plotted against corresponding values of -k. The correlation was highly significant, with the correlation coefficient $r_{61} = + 0.860$, $p < 0.001$. Δ and -k were related by $\Delta = 0.63\ (-k) + 0.16$. The standard error of estimate of Δ was 18.8%. The correlation was improved when mean values of Δ and -k were taken, and Fig. 27 shows a plot of mean values for 44 patients. The correlation coefficient $r_{44} = + 0.894$, $p < 0.001$. Δ and -k were related by $\Delta = 0.67\ (-k) + 0.07$. The standard error of estimate of Δ was 15.1%.

The 11 specimens from patients with proteinuria of under 1 gm./day have been analysed separately and form a quite different statistical population. The correlation is shown in Fig. 28. Values of Δ, when compared to values of -k, are much lower than for patients with proteinuria of over 1 gm./day and the correlation between Δ and -k is not statistically significant ($r_{11} = + 0.469$).

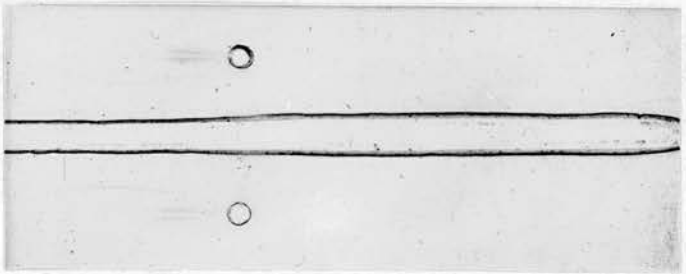
Immunoelectrophoretic examination of serum and urine elution patterns on Sephadex has already been described. These results confirmed that urine and serum proteins, which were not only of the same molecular weight but were also antigenically alike, were being compared when selectivity values were estimated by gel filtration.

Fig. 29. Identification of orosomucoid component in the antiserum.

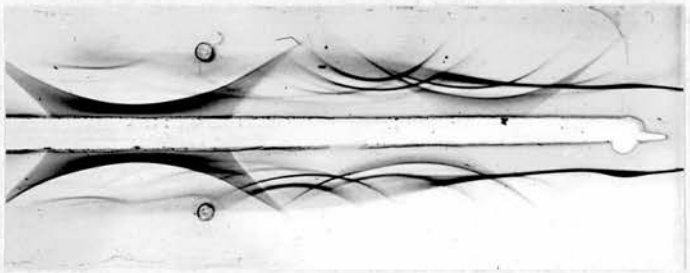
a. Serum reacted against anti-orosomucoid serum



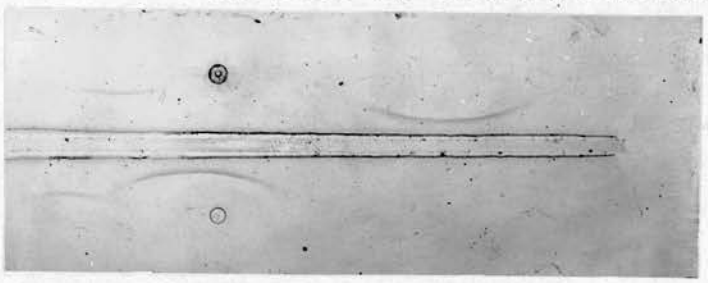
b. Serum reacted against anti-orosomucoid serum which had been absorbed with albumin. Component (b) could not be detected.



c. Serum which had been incubated with neuraminidase (NS) and a control serum (CS) reacted against anti-human serum. The mobility of some of the NS proteins was affected.

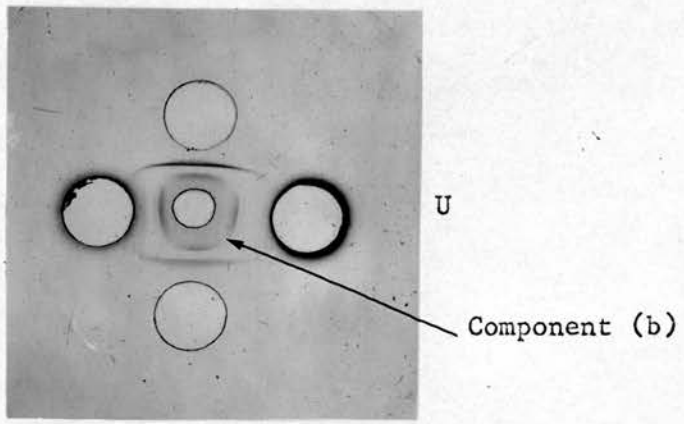


d. Serum which had been incubated with neuraminidase (NS) and a control serum (CS) reacted against anti-orosomucoid serum. The mobility of component (a) of the serum was markedly affected.



S

e. Serum (S) and urine (U) reacted against anti-orosomucoid serum. Only component (b) was detected in the urine.



S

3.1.2. CLEARANCES OF OTHER INDIVIDUAL PROTEINS AND ENZYMES.

The relative clearances of six other individual proteins and four enzymes were studied in relation to selectivity, in a number of different types of renal disease.

Orosomucoid

The antisera to orosomucoid was found in several batches to be specific for two components when examined by immunoelectrophoresis (Fig. 29a). The corresponding proteins migrated to the albumin-α_1-region on electrophoresis. Both precipitin arcs stained for glycoprotein, neither stained for lipoprotein. It was thought that the faster diffusing component (a), which precipitated closer to the antisera trough, might be albumin; absorption of the antisera with albumin tended to support this (Fig. 29b), and morevoer albumin is a likely contaminating antibody for orosomucoid. Addition of anti-albumin serum to the anti-orosomucoid serum did not produce a third component. If (a) was albumin, the more slowly diffusing component (b) would then be orosomucoid.

Fractions of normal serum obtained by gel filtration were examined by immunoelectrophoresis against anti-orosomucoid serum. Protein (a) was found to elute in the third peak with the 4S material; protein (b) was found to elute at the first trough and second peak with the 11S and 7S material. Again, this was compatible with component (a) being albumin. However, orosomucoid is a much smaller protein than (b), as suggested by gel filtration of (b); although carbohydrate rich proteins are known to behave anomalously on Sephadex (Andrews, 1965). The slow diffusion of (b) in agar also indicated a high molecular weight, but orosomucoid has been reported to polymerize (Whitehead, 1965), so that the possible identity of orosomucoid with (b) could not be excluded. On the other hand the results equally

suggested that (a) was orosomucoid and that the elimination of (a) on absorption with albumin was due to contamination of the albumin with orosomucoid.

In order to resolve this problem, 1 ml. of normal serum was incubated with 0.5 mg. neuraminidase at pH 7.0 for 24 hours and afterwards reacted immunoelectrophoretically against anti-orosomucoid and anti-human serum. Neuraminidase splits peripheral sialic acid groups from proteins, and therefore can reduce the mobility of glycoproteins towards the anode (Schultze, 1962). Results are shown in Figs. 29c and d. The control was incubated at pH 7.0 for 24 hours. It can be seen that neuraminidase treatment slightly altered the electrophoretic mobility of several of the serum proteins; these were proteins which have a high carbohydrate content. However the most dramatic change was seen against the anti-orosomucoid serum. Component (a) disappeared from the α_1-region, and instead a precipitin arc formed in the β-region; component (b) was relatively unaffected. Since orosomucoid has a high sialic acid content, this provided direct evidence that component (a) was orosomucoid and that the high molecular weight component (b) was the contaminating antibody.

On immunodiffusion of serum against anti-orosomucoid two hexagons were seen around each serum antigen hole, corresponding to components (a) and (b). The faster diffusing component (a) formed the outer hexagon; this was therefore read against the urine hexagons, although with difficulty as the precipitin lines tended to be rather faint. Urinary orosomucoid was difficult to detect, and in some cases no precipitation could be obtained over a wide range of urine concentrations, including the urine concentrate. In other cases precipitation was only obtained using the concentrate. Some clearance values for five patients in relation to the other proteins are

TABLE 22.

Relative clearances of six other individual proteins

Clearances of orosomucoid (O), prealbumin (p), ceruloplasmin (C), γ_{1A}-globulin (γ_{1A}), α_1-lipoprotein, (α_1) and fibrinogen (F) in relation to the five clearances normally estimated for selectivity determinations, albumin(100%), transferrin (S), γ-globulin (γ), α_2-macroglobulin (α_2) and β-lipoprotein (β).

Subject	O	p	S	γ	C	γ_{1A}	α_1	F	α_2	β
1	3.2	72	72	21	50				3.5	0.02
2	41	40	80	17	14				0.72	
3a	1.7	25	75	15					0.60	0.05
4	8.4	70	11						0.30	
5a	6.3	70	100	35					0.35	
6a		72	125	18	36				0.70	0.05
7a		25	86	19	37				1.9	0.15
8		63	100	31	31				3.1	0.75
9a		38	86	75					12.0	3.2
10a		21	100	100	54				25.0	
11		43	86	38					19	2.9
5b		44	100	36					0.71	
12a		50	67	20	50				1.8	0.16
13		35	70	15	15		2.2		14	0.23
14		200	114	29	50	40	25		0.89	0.04
15		14	36	7.8	2.5	6.3	3.2		0.06	
16		20	57	13	33	20	6.3		0.78	0.13
12b		37	150	43	60	30	9.4		2.3	0.42
17			72	3.1		8.9			13	
6b			100	29	29	14	10		0.73	0.21
18			44	25	25	22	18		11	3.3
19			100	3.9	28	1.6	2.3		0.01	
20			70	22	34	9.9	2.1		1.5	
3b			57	13	51	11	1.8		0.80	
21			100	7.1	21	2.1	1.1		0.03	
7b			70	29	50	25	0.65		2.7	0.35
22			100	70	87	58	25		6.3	1.7
23			117	8.8				0.10	0.19	0.05
24			125	25				0.10	1.9	
25			88	44				1.9	0.03	
26			120	19				0.15	0.97	
27			67	20				0.13	0.76	
28			125	5.0				0.18	11	1.2
29			44	4.4				0.29	0.44	0.29
30			86	13				0.71	4.1	0.64

given in Table 22. The relative clearances of orosomucoid are anomalously low, considering the molecular weight (44,000). Cross reactions were tested for, using serum and urine concentrate against anti-orosomucoid. As suspected, it was found that the serum orosomucoid was being read against the urine high molecular weight component (b), (Fig. 29e), thus accounting for the low clearance values.

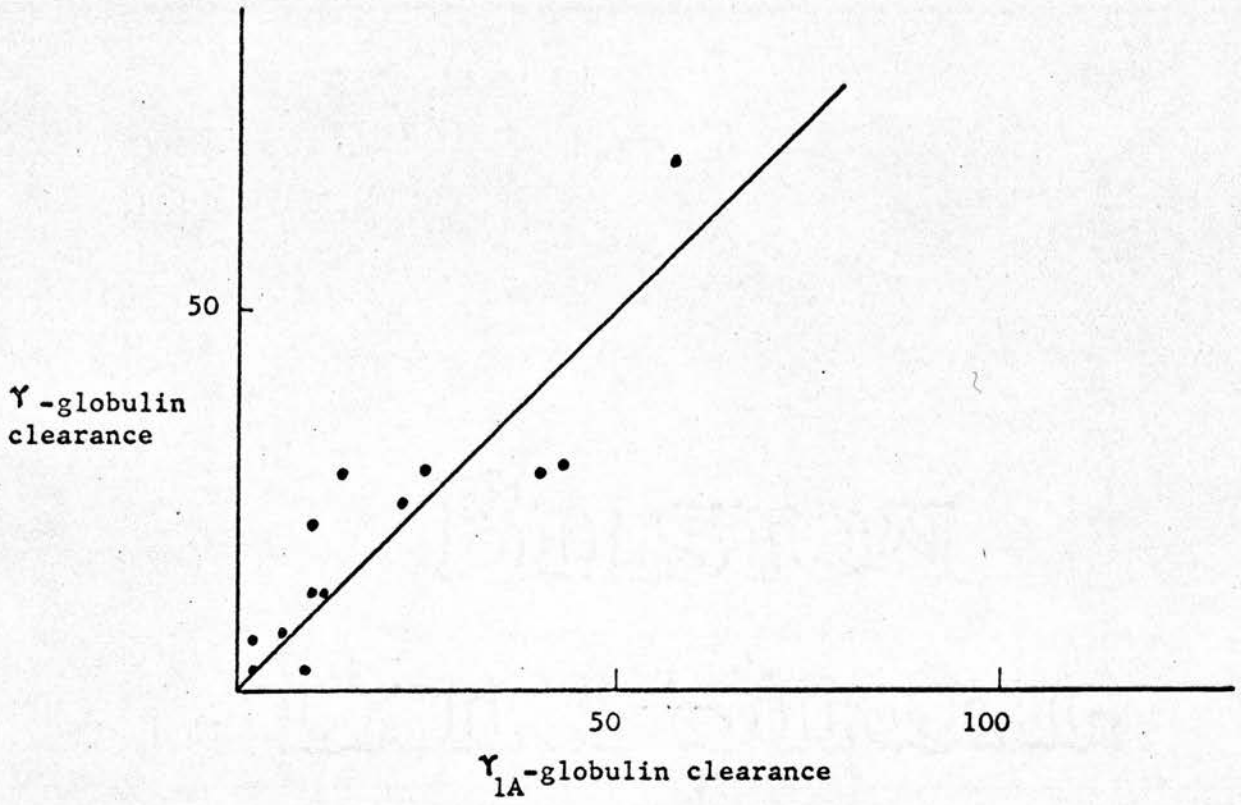
Urine from patients with the nephrotic syndrome contains relatively high concentrations of orosomucoid (Popenhoe,1955). The apparent absence of orosomucoid in the urines studied could only be accounted for by the fact that the orosomucoid component of the antiserum was relatively weak, as suggested by the faintness of the serum hexagons. This was also indicated on immunoelectrophoresis of normal serum against anti-orosomucoid serum diluted x 2, when only the contaminating high molecular weight component (b) was seen.

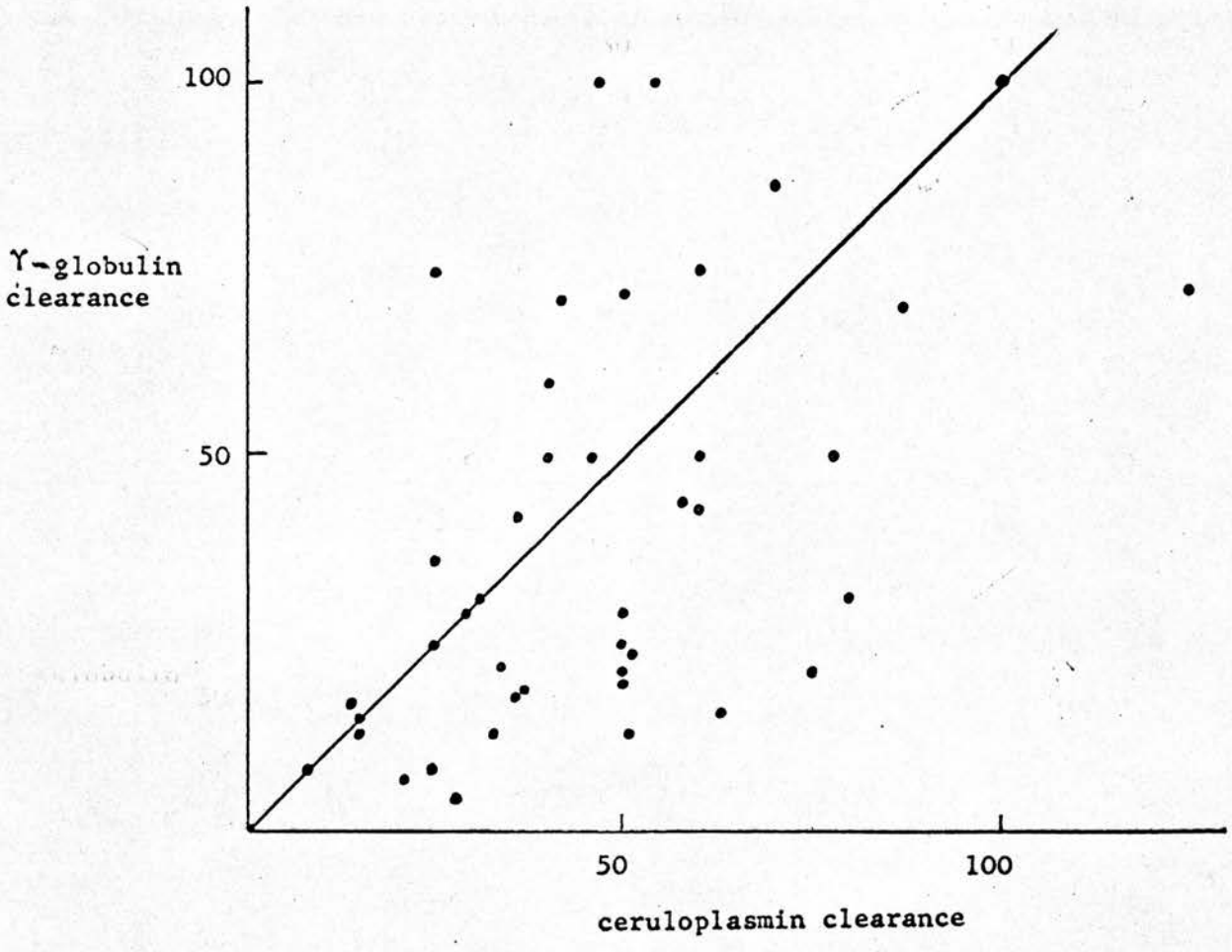
In a few cases a careful search for urinary orosomucoid was carried out. The expected clearance of orosomucoid was calculated from the selectivity. Serum and appropriate urine dilutions were then set up on an immunodiffusion plate, against a stronger series of dilutions of anti-orosomucoid. After addition of 2% acetic acid to the plates some faint precipitin arcs could be seen, particularly against the two weakest antisera dilutions. This confirmed that orosomucoid was present in the urines examined, and at the expected dilutions, but it was not found possible to estimate the urine:serum ratios.

Prealbumin

Clearances of prealbumin were estimated 18 times on 16 patients and were found, with one exception, to be lower than the corresponding albumin clearances. Table 22 gives some estimations of the relative prealbumin

Fig. 30. Correlation of γ-globulin clearances with clearances of γ_{1A}-globulin and ceruloplasmin.





clearances in relation to the relative clearances of other proteins. Values ranged from 14 - 200%.

Since prealbumin (mol. wt. 61,000) is a slightly smaller molecule than albumin these clearance figures were not consistent with the clearance values of other proteins. Therefore no attempt was made to estimate the effect of the prealbumin clearances on the selectivity index.

Ceruloplasmin and Υ_{1A}-globulin

Ceruloplasmin clearances were estimated 42 times on 30 patients. Values ranged from 8.3 - 125% of the albumin clearance. Clearances of Υ_{1A}-globulin were estimated on 13 patients and values ranged from 2.1 - 58% of the albumin clearance. Some of the results for both proteins are shown in relation to the clearance of other proteins in Table 22.

Since both ceruloplasmin (mol. wt. 150,000) and Υ_{1A}-globulin (mol. wt. 160,000) have similar molecular weights to that of Υ-globulin a direct comparison of the clearance values was carried out. Fig. 30 shows the Υ_{1A}-globulin clearance and the ceruloplasmin clearance plotted against the Υ-globulin clearance and in both cases there was a positive correlation. The value of the correlation coefficient for ceruloplasmin and Υ-globulin, $r_{42} = +0.512$, $p < 0.001$, and for Υ_{1A}-globulin and Υ-globulin, $r_{13} = +0.901$, $p < 0.001$. The ceruloplasmin clearance tended to be higher than the Υ-globulin clearance, but the mean values, 47 and 40% respectively, were not significantly different. The Υ_{1A}-globulin clearance tended to be lower than the Υ-globulin clearance, but again the mean values, 19 and 23% respectively, were not significantly different.

Selectivity values were calculated with and without including the clearances of ceruloplasmin and Υ_{1A}-globulin (Table 23). The index of selectivity was not affected. The coefficients of variation calculated from

TABLE 23.

Selectivity values with and without including clearances of other individual proteins

Values of selectivity calculated on the basis of the clearances of albumin, transferrin, γ-globulin, α_2-macroglobulin and β-lipoprotein compared to values calculated on the basis of these five proteins and the clearance(s) of one or more additional proteins (ceruloplasmin (C), γ_{1A}-globulin (γ_{1A}) α_1-lipoprotein (α_1), and fibrinogen (F).

Subject	Selectivity values					
	5 proteins	+C	$+\gamma_{1A}$	$+\alpha_1$	$+C,\gamma_{1A},\alpha_1$	+F
13	1.67	1.65		1.61		
14	2.22	2.25	2.24	2.23	2.27	
15	2.93	2.96	2.93	2.94	2.97	
16	1.83	1.86	1.84	1.81	1.85	
12b	1.64	1.66	1.63	1.61	1.63	
17	1.71		1.71			
6b	1.85				1.82	
18	0.82	0.80	0.85	0.80	0.79	
19	3.74				3.77	
20	1.69				1.78	
3b	1.92				2.02	
21	3.40				3.45	
7b	1.55				1.51	
22	1.20	1.23	1.21	1.19	1.22	
23	2.26					2.30
24	1.69					2.29
25	3.42					3.33
26	1.97					2.45
27	1.97					2.45
28	1.22					1.28
29	2.10					2.38
30	1.33					1.37

seven pairs of values were 0.9% and 0.5% for ceruloplasmin and γ_{1A}-globulin respectively.

α_1-Lipoprotein

Clearances of α_1-lipoprotein were estimated on 13 patients. Values ranged from 0.65 - 25% of the albumin clearance. The results are shown in relation to the clearance of the other proteins in Table 22.

The α_1-lipoprotein molecule (mol. wt. 200,000) is slightly larger than that of γ-globulin, but is smaller than α_2-macroglobulin. With one exception, the clearance values for α_1-lipoprotein were between the values for γ-globulin and α_2-macroglobulin. Selectivity values calculated with and without including the α_1-lipoprotein clearance showed no significant difference (Table 23). The coefficient of variation calculated from seven pairs of values was 1.1%.

Fibrinogen

Clearances of fibrinogen were estimated on 8 patients. Values ranged from 0.10 - 1.9% of the albumin clearance. The results are shown in relation to the clearance of the other proteins in Table 22.

Fibrinogen (mol. wt. 330,000) is a larger molecule than γ-globulin, but smaller than α_2-macroglobulin. However the clearances were generally less than those of α_2-macroglobulin. Selectivity values calculated with and without including the fibrinogen clearance showed a significant difference (Table 23). The coefficient of variation calculated from six pairs of values was 11.3%.

Amylase

Serum and urine amylases were estimated 20 times on 15 patients, all of whom had significant proteinuria. The serum levels ranged from 45 - 167 units/ml., mean 122 units/ml. and urine values from 970 - 210,000 units/24 hr., mean 122.850 units/24 hr. These units are similar to Somogyi units, and

TABLE 24.

Amylase clearances

Subject	Serum units/ml.	Urine units/ml.	Serum: urine	Urine vol. ml.	Urine units./24 hr.	Albumin serum: urine	-k	Predicted amylase clearance	Actual amylase clearance	Creatinine clearance ml./min.	Urine protein g./24 hr.
2	88	144	0.61	540	77,700	0.375	3.07	309	163	60	28.5
	85	159	0.54	1320	210,000	0.0625	2.84	288	12	37	19.8
1	103	73	1.4	1450	106,000	48	3.54	372	3400	97	3.5
3	154	71	2.2	1740	123,500	40	2.74	275	1840	38	3.1
4	163	103	1.6	1900	196,000	3	2.74	275	190	33	17.3
5	150	118	1.3	890	105,000	128	2.48	251	10100	68	0.3
6	83	131	0.63	1210	159,000	2	2.36	240	315	168	8.5
7	167	237	0.71	1140	270,000	64	2.00	209	9100	123	0.7
8	87	96	0.91	1190	114,000	5	1.72	191	550	49	5.5
	45	55	0.82	1400	77,000	3.5	1.56	178	437	22	16.8
9	163	174	0.94	1050	183,000	3.5	1.69	186	374	55	6.3
	167	102	1.6	1240	126,000	5	1.67	185	305	38	2.4
10	99	73	1.4	2295	168,000	24	1.67	185	1775	50	2.3
	132	76	1.7	810	6,150	40	1.28	162	2300	49	1.5
11	144	152	0.95	1350	205,000	5	1.61	182	528	99	6.8
12	136	176	0.78	1070	188,000	3	1.31	162	388	54	9.6
13	121	85	1.4	1170	99,500	4	1.09	150	280	18	5.9
14	74	23	3.2	42	970	80	0.41	117	250	7	1.2
	120	92	1.3	265	2,440	14	0.31	114	1080	3	1.2
15	150	17	8.8	2335	41,600	560	-	-	6360	65	0.3

normal serum and urine values in Somogyi units have been given as follows:

Serum 60 - 160 units/ml. (Van Loon, Likins and Seger, 1952)

38 - 191 units/ml. (Saxon et al., 1957)

Urine 1,200 - 8,580 units/24 hr. (Smith and Roe, 1952)

792 - 4,264 units/24 hr. (Saxon et al., 1957)

The serum values in the present series therefore were within normal limits, but the urine values were significantly raised.

Urine:serum ratios were calculated for amylase and the clearance expressed as a percentage of the albumin clearance. This value was then compared to a predicted value calculated from the index of selectivity, assuming the relative amylase (mol. wt. 45,000) clearance fell on the linear log-log plot, whose slope is the index of selectivity. Results are shown in Table 24, which also gives the serum and urine amylase values, creatinine clearance and total urine protein. Actual amylase clearances were generally much higher than the predicted clearance, and in only 11 cases were the actual and predicted values of the same order of magnitude. There was no overall correlation between relative amylase clearance and selectivity. There was, however, a correlation between amylase excretion and total protein excretion, correlation coefficient $r_{20} = + 0.599$, $p < 0.01$.

Pepsinogen

Serum and urine pepsins were estimated 15 times on 12 patients, all of whom had significant proteinuria. Results were expressed both as Hunt units (an arbitrary unit based on a phenol standard), and as μg. tyrosine liberated from 1 ml. of sample in 24 hr. Serum levels were probably within normal limits (Hanley, 1964; Singh and Shinton, 1965). Normal urinary pepsin values have been expressed in a variety of units by different workers. Comparison of the present series with other results is therefore difficult,

TABLE 25.

Pepsin(ogen) clearances

Subject	Hunt units			Tyrosine units			Urine vol.	Urine units/ 24 hr.		Albumin serum: urine	-k	Predicted Pepsinogen clearance	Actual Pepsinogen clearance		Creat. clear. ml/min.	Urine protein g/24hr.
	Serum/ ml.	Urine/ ml.	Serum: urine	Serum/ ml.	Urine/ ml.	Serum: urine		Hunt	Tyrosine $x10^{-3}$				Hunt units	Tyrosine units		
1	1.85	22.5	0.082	140	940	0.15	1450	32,600	1360	48	3.54	468	58,500	32,200	97	3.5
2	2.60	47.0	0.056	200	1425	0.14	540	25,400	770	0.375	3.07	380	675	268	60	28.5
	2.05	15.3	0.13	160	745	0.22	1320	20,100	985	0.0625	2.84	347	48	29	37	19.8
3	7.7	31.5	0.25	470	1135	0.41	1740	54,800	1975	40	2.74	331	16,300	9,680	38	3.1
4	1.75	30.8	0.057	140	1120	0.13	890	27,300	996	128	2.48	295	225,000	102,500	68	0.3
5	2.85	62.5	0.045	210	1635	0.13	1210	75,600	1980	2	2.36	282	4,450	1,550	168	8.5
6	9.8	67.0	0.15	560	1720	0.33	1190	79,600	2050	5	1.72	214	3,320	1,535	49	5.5
	4.6	18.5	0.25	325	835	0.39	1400	25,900	455	3.5	1.56	200	1,400	900	22	16.8
7	4.9	8.75	0.56	345	525	0.66	1240	10,750	650	5	1.67	209	895	760	38	2.4
8	2.70	61.0	0.038	200	1615	0.12	1350	82,500	2180	5	1.61	204	13,150	4,030	99	6.8
9	10.0	19.8	0.51	570	870	0.66	1120	22,200	975	40	1.44	186	7,900	6,100	5	-
10	1.60	44.5	0.036	135	1380	0.098	1070	47,600	1475	3	1.31	178	8,350	3,070	54	9.6
11	1.25	16.5	0.076	105	800	0.13	810	13,400	648	40	1.28	174	52,500	30,500	49	1.5
12	1.95	3.6	0.54	150	255	0.59	42	151	11	80	0.41	120	14,800	13,600	7	1.2
	4.0	41.3	0.097	285	1325	0.22	265	10,900	351	14	0.31	115	14,500	6,520	3	1.2

but adaption of values obtained by Van Goidsenhoven, Wilkoff and Kirsner (1958) and Mackenzie (1953) suggest that the urinary levels were not abnormal.

Urine:serum ratios were calculated for pepsinogen (mol. wt. 48,000) and the relative clearances compared to the predicted clearance, as for amylase. Results are shown in Table 25. The relative clearances were consistently lower when the values were expressed as tyrosine, as opposed to Hunt units. However, in neither case was there any correlation between actual and predicted pepsinogen clearance. The actual clearances were generally of the order of 100 times the predicted clearances. There was no correlation between pepsinogen excretion and total protein excretion, correlation coefficient, $r_{14} = -0.140$.

Lactic dehydrogenase(LD)

Serum and urine LDs were estimated 20 times on 18 patients, 14 of whom had significant proteinuria. The serum levels ranged from 143 - 340 units/ml., mean 210 units/ml. and the urine levels from 8,500 - 216,000 units/24 hr., mean 41,000 units/24 hr. Normal serum and urine values have been given as follows:

Serum 260 - 850 units/ml. (Wroblewski and LaDue, 1955)
150 - 500 units/ml. (Rosalki and Wilkinson, 1959)

Urine 7,000 - 32,000 units/24 hr. (Rosalki and Wilkinson, 1959)
1,225 - 6,090 units/24 hr. (Wacker and Dorfman, 1962).

The serum values in the present series were therefore within normal limits, although the distribution was towards the lower limit of normal. Urine values were significantly raised.

Urine:serum ratios were calculated for LD (mol. wt. 136,000), and the relative clearances compared to the predicted clearances as for amylase.

TABLE 26.

LD clearances

Subject	Serum units/ml.	Urine units/ml.	Serum: urine	Urine vol. ml.	Urine units/24 hr.	Albumin serum: urine	-k	Predicted LD clearance	Actual LD clearance	Creatinine clearance ml./min.	Urine protein g./24 hr.
1	190	114	1.7	1900	216,000	0.625	2.61	17	36	27	12.9
2	200	32	6.3	1670	53,500	2.5	2.38	20	40	33	13.4
3	190	24	7.9	1210	29,100	2.0	2.36	20	25	168	8.5
4	125	11	12	1140	12,000	64	2.00	25	538	123	0.7
5	165	7.8	21	1750	13,650	6	1.96	27	28	125	6.1
6	245	5	49	2000	10,000	32	1.90	28	65	63	3.2
7	280	53	5.3	1050	55,700	3.5	1.69	32	66	55	6.3
	215	23	9.4	245	28,600	5	1.67	33	37	38	2.4
8	340	7.8	44	2295	17,900	24	1.67	33	55	50	2.3
9	320	17	19	1350	23,000	5	1.61	34	26	99	6.8
10	160	23	7.1	925	20,800	8	1.51	36	112	8	6.5
11	155	81	1.9	293	23,700	1.25	1.39	40	65	66	6.9
	205	40	5.1	1070	43,800	3	1.31	43	59	54	9.6
12	185	20	9.3	2400	48,000	28	1.39	40	302	19	5.3
13	175	16	11	2120	34,000	16	1.34	41	142	10	4.7
14	285	40	7.1	1170	46,800	4	1.09	48	56	18	5.9
15	180	6.5	28	1370	8,900	896	(0.97)	(52)	295	139	-
16	258	6.5	40	1310	8,500	2048	(1.44)	(38)	476	7	-
17	143	27	5.3	1210	32,700	320	-	-	6150	103	18.4
18	185	6.8	27	3540	95,400	560	-	-	2070	128	0.3

Results are shown in Table 26, which also gives serum and urine LD values, creatinine clearance and total urine protein. Of the 14 patients with proteinuria, 10 had clearances which had a correlation with the predicted clearance, the correlation coefficient $r_{10} = + 0.539$, $p < 0.05$. In these 10 cases there was no significant difference between indices of selectivity calculated with and without including the LD clearance. The coefficient of variation for the 10 pairs of values was 1.4%. In the other 4 patients with proteinuria the LD clearances were all significantly higher than the predicted values. In 3 of these patients there was a large amount of urinary LD, and the proteinuria was unselective. In the fourth patient the proteinuria was under 1 gm./day and the serum LD was low. In the 4 patients with normal or trace proteinuria the LD clearances were also higher than the predicted values; in these cases the high urinary levels contributed to the high clearance values.

LD excretion had a correlation with the total protein excretion, correlation coefficient $r_{15} = + 0.611$, $p < 0.05$.

Glutamic oxalacetic transaminase (GOT)

Serum and urine GOTs were estimated 11 times on 10 patients, 9 of whom had significant proteinuria. The serum levels ranged from 24 - 52 units/ml., mean 40 units/ml. and the urine levels from 3,570 - 26,100 units/24 hr., mean 10,770 units/24 hr. Normal serum and urine values have been given as follows:

Serum 5 - 35 units/ml. (Rosalki and Wilkinson, 1959)

7 - 40 units/ml. (Chinsky, Shmagranoff and Sherry, 1956)

Urine 5000 units/24 hr. (Rosalki and Wilkinson, 1959)

30% of serum value (Sephaha, Bhandari and Visayvargiya, 1961)

The serum values in the present series were therefore within normal limits,

TABLE 27.

GOT clearances

Subject	Serum units/ml.	Urine units/ml.	Serum: urine	Urine vol. ml.	Urine units/24 hr.	Albumin serum: urine	-k	Predicted GOT clearance	Actual GOT clearance	Creatinine clearance ml./min.	Urine protein g./24 hr
1	45	6.3	7.1	1450	9,150	112	3.95	16	1580	173	-
	52	8.8	5.9	2970	26,100	10	3.55	20	170	86	5.9
2	30	3.4	8.8	1050	3,570	12	2.87	27	137	96	2.8
3	39	8.0	4.9	750	6,000	48	2.54	31	980	64	-
4	46	19.4	2.4	730	14,150	0.975	2.36	34	41	30	6.1
5	48	5.2	9.2	1450	7,300	32	1.76	45	348	129	2.3
6	40	5.0	8.0	2300	11,500	16	1.70	46	200	39	5.9
7	24	2.5	9.6	1450	3,630	56	1.62	48	585	110	1.3
8	45	12.0	3.8	1010	12,100	3.5	1.60	48	92	150	8.1
9	30	8.3	3.6	1250	10,350	8	0.98	63	222	17	5.5
10	41	2.8	14.6	1400	3,820	1280	-	-	8800	39	-

although the distribution was towards the upper limit of normal. Urine values were raised.

Urine:serum ratios were calculated for GOT (mol. wt. 110,000), and the relative clearances compared to the predicted clearances as for amylase. Results are shown in Table 27, which also gives the serum and urine LD values, creatinine clearance and total urine protein. The actual GOT clearances were significantly higher than the predicted clearances, particularly when the level of urinary protein was low. There was no correlation between GOT clearance and selectivity. There was a degree of correlation between urinary GOT and urinary protein, $r_g = + 0.635$, $p < 0.1$.

Summary

Serum levels of the four enzymes studied were probably not abnormal. Urinary amylase, LD and GOT values were significantly raised, urinary pepsinogen values were probably normal.

Overall, the clearance values did not correlate with values predicted from the index of selectivity. Clearances of amylase were higher than the predicted values, although some were of the same order of magnitude. Clearances of pepsinogen were of the order of 100 times the predicted value. Clearances of LD, however, did correlate with selectivity in 10 out of 14 patients. In the other 4 patients clearances were significantly higher than the predicted values. Clearances of GOT were significantly higher than predicted values.

Urinary enzyme levels had a correlation with total protein excretion in the case of amylase, LD, and GOT.

Of the clearances of six other individual proteins estimated by immunodiffusion, only prealbumin and fibrinogen did not fit the linear relationship of renal clearance of protein and molecular weight on a log-log

scale. Clearances of γ_{1A}-globulin, ceruloplasmin, and α_1-lipoprotein were found to correlate well with selectivity; calculation of the index of selectivity including these proteins did not affect the values. Clearances of orosomucoid were technically difficult to measure, but the values probably approximated to the expected values. Clearances of prealbumin and fibrinogen were lower than predicted, although values for both proteins were not of a different order of magnitude.

TABLE 28.

Dextran indices of selectivity in renal disease

Dextran indices of selectivity (D) were estimated in 22 patients with different types of renal disease. The creatinine clearance (Cr Cl), total urine protein (UP)are also shown, and in two cases the albumin serum:urine ratio (Alb S:U) is given.

Subject	D	UP g./24 hr.	Cr Cl ml./min.	Diagnosis
1	2.65	0.78	10	Acute ischaemic renal failure
2	2.46	37 mg.	175	Recovered minimal lesion GN
3	2.12	7.5	22	Amyloid disease
4	2.05	4.4	9	Chronic renal failure
5	2.03	4.5	77	Proliferative glomerulonephritis
6	2.02	-	30	Acute ischaemic renal failure
7	1.99	0.62	7	Acute " " "
8	1.99	4.8	122	Minimal lesion glomerulo-nephritis
9	1.95	14.9	97	Minimal " "
10	1.90	(Alb S:U = 380)	134	Proliferative glomerulonephritis
2	1.86	10.0	32	Minimal lesion glomerulo-nephritis
11	1.83	4.8	110	Minimal " "
12	1.81	6.8	168	Proliferative "
13	1.75	11.7	66	Mixed M and P "
14	1.68	11.8	5	Amyloid disease
15	1.62	13.4	33	Proliferative glomerulonephritis
16	1.59	6.8	99	" "
17	1.53	6.6	38	Membranous "
18	1.51	19.6 (Alb S:U = 320)	5	Myelomatosis
19	1.45	3.0	27	Lupus nephritis
20	1.17	9.0	7	Membranous glomerulonephritis
21	1.17	8.4	53	Proliferative "

TABLE 29.

Dextran Infusion Experiments

Values of dextran selectivity (D) were estimated after infusion of dextran. Details are given of samples taken, creatinine clearance (Cr Cl), dextran excreted and protein excreted (UP) and protein selectivity (-k).

Subject	Time of infusion	amount of dextran given	Serum samples	Urine samples	Urine vol. ml.	Urine flow ml/min.	Cr Cl ml/min	Dextran excreted mg/ml.	Dextran excreted mg/min.	Total Dextran excreted mg.	D	UP g/24 hr.	-k
	5.05-5.10 p.m.						10						
1		33 g.	5.15 pm	5.15-8.15	380	2.11	14	6.8	14.2	2580	2.91		
			8.15 pm	8.15-8.20	8			8.6		69	2.67	0.78	
				8.20-9.00	930	1.23	8	3.6	4.4	3350	2.81		
			9.00 am										
	11.00-11.15 am		11.30 am				7.3						
				11.30-12.10	35	0.88	8.3	4.8	4.2	166	1.82		
2		22 g.	12.10 am										
				12.10-1.00	25	0.50	4.5	5.9	3.0	147	2.13	0.62	
			1.00 pm										
				1.00-2.00	68	1.13	9.8	5.4	6.1	368	2.11		
			2.00 pm										
	2.30-2.50 pm						32						2.48
			2.50 pm										
				2.50-3.10	16	0.80	34	15.8	12.6	253	1.75		
3		23 g.	3.10 pm										
				3.10-3.40	31	1.03	45	20.6	21.2	639	1.84	10.0	2.61
			3.40 pm										
				3.40-4.00	126	63	72	5.0	31.5	630	2.00		
			4.00 pm										
	2.15-2.30 pm						6.5						1.42
			2.40 pm										
				2.40-3.25	335	7.44	15	4.6	34.6	1540	1.12		
			3.25 pm										
4		22 g.		3.25-4.10	305	6.79	13	3.8	25.8	1090	1.36	9.0	1.34
			4.10 pm										
				4.30-9.00	1250	1.25	8	6.6	8.2	825	1.30		
			9.15 am										

3.1.3. DEXTRAN STUDIES

Selectivity values

Selectivity values were estimated on 21 patients, with both trace proteinuria and significant proteinuria, and with several different types of renal disease. Values ranged from 1.17 - 2.65, mean 1.79. Results are given in Table 28, which also gives the total protein excretion, creatinine clearance and diagnosis.

Results of the four very carefully controlled infusion experiments are given in Table 29. In every subject the creatinine clearance was higher immediately post-infusion than pre-infusion. In subjects 1 and 4 the value for the final collection period had fallen to near the pre-infusion value. In subject 2 the creatinine clearance fell during the second collection period and rose again over the final collection period, but the values were very low and the differences were probably not significant. In subject 3 the creatinine clearance rose over the second collection and rose again markedly over the final collection period. This last rise was undoubtedly due to the wash-out effect of the diuretic frusemide which was given.

Dextran selectivity values also showed variations over the collection periods. In subject 3 there was a steady rise, from first to second to third value; the difference between the first and third values was 10.4%. In subjects 2 and 4 the second values were slightly higher than the third, but the third values were 12.5% and 16.1% respectively higher than the first value. In subject 1 the selectivity fell and then rose again; the third value was 5.2% lower than the first. Since the error of the method is about $\pm$ 10% these variations are probably not significant. However, in the 4 subjects, the mean variation in dextran selectivity over all the collection periods was + 8.2%. No significant change in protein selectivity

TABLE 30.

Dextran selectivity over a lower molecular weight range

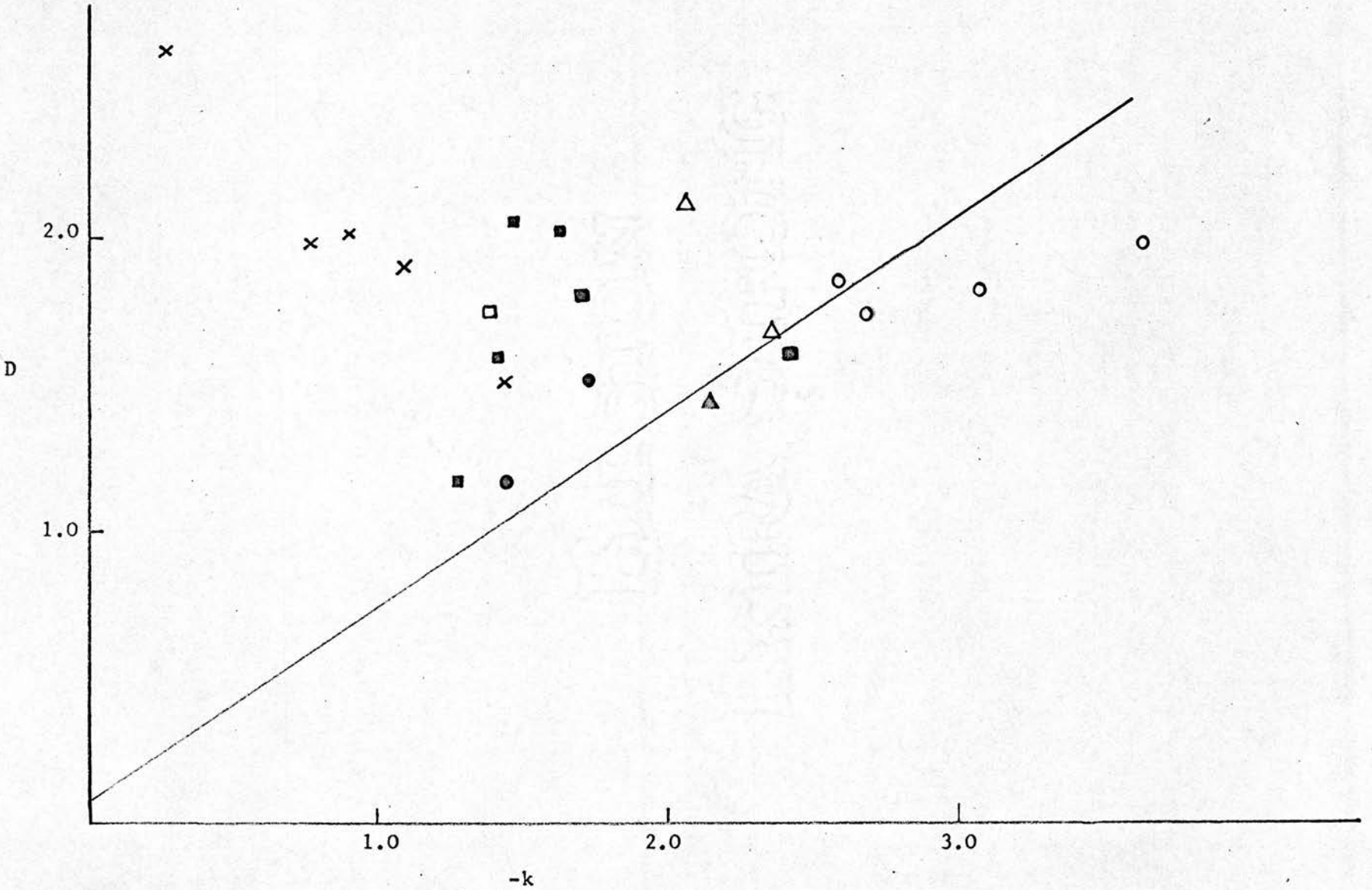
Indices of dextran selectivity (D) were calculated from the slope of the line relating relative clearance to molecular weight on a log-log scale, between the 1st and 3rd serum protein peaks on G 200. The slope of this line, for a further 10 tubes towards a lower molecular weight range, was then estimated and a corresponding value of D calculated. The percentage change in slope is given.

Subject	Slope 1st - 3rd protein peaks x 100	Corresponding D	Slope for further 10 tubes x 100	Corresponding D	% change in slope
1	5.92	1.30	4.20	0.92	29
2	7.29	1.82	5.37	1.34	26
	8.90	2.11	3.27	0.79	63
3	8.00	1.84	6.27	1.44	22
4	9.82	2.46	8.78	2.20	10
5	9.07	2.18	5.09	1.22	43
6	9.31	2.34	5.25	1.31	44

Fig. 31. Correlation of dextran and immunodiffusion protein indices of selectivity.

The line represents the expected correlation and is the calculated regression line from immunodiffusion and gel filtration protein studies.

○ minimal lesion glomerulonephritis, ● membranous glomerulonephritis, △ amyloid disease, ▲ lupus nephritis, ■ proliferative glomerulonephritis, □ mixed membranous and proliferative glomerulonephritis, × minimal proteinuria.



was noted.

The slopes of the dextran log-log plots of relative clearance against molecular weight were also calculated over a lower molecular weight range, in some of the cases where the urine dextran had not been concentrated. The results are shown in Table 30. In every case there was a significant reduction in slope, the changes ranging from 10 - 63%.

The dextran clearance was compared to the creatinine clearance in 5 experiments. At the third (4S) protein peak on Sephadex the dextran clearance averaged 13% of the creatinine clearance, 9 - 10 tubes and 14 - 15 tubes later (elution volumes of a further 18 - 20 ml. and 28 - 30 ml. respectively) the values were 39% and 59% of the creatinine clearance respectively.

Correlation of dextran and protein indices of selectivity

Dextran indices of selectivity were compared with protein indices obtained by both immunodiffusion and gel filtration in 21 patients.

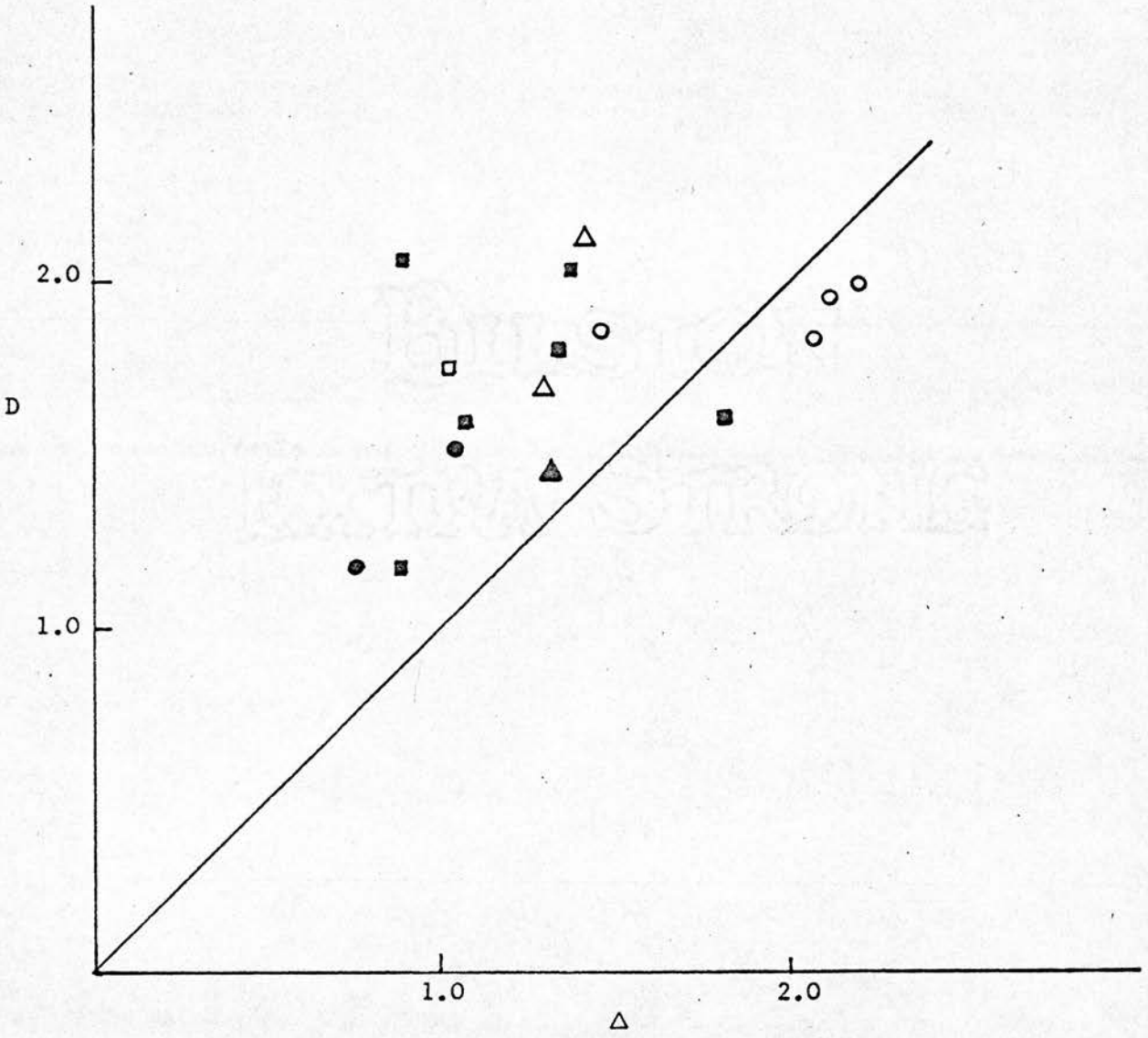
Fig. 31 shows dextran indices of selectivity plotted against protein indices estimated by immunodiffusion, and Fig. 32 shows dextran indices plotted against protein indices estimated by gel filtration. The diagnosis is also indicated. There is no correlation between dextran and protein indices of selectivity. Although the dextran selectivity values were within the range of protein selectivity values, they were generally significantly higher. This was particularly marked in the group of patients with proteinuria under 1 gm./day (Fig. 31). This included 3 patients with acute ischaemic renal failure, 1 with acute proliferative glomerulonephritis and 1 with multiple myeloma. Protein indices of selectivity by gel filtration were not estimated in these patients.

Patients with minimal lesion glomerulonephritis and membranous

Fig. 32. Correlation of dextran and gel filtration protein indices of selectivity.

The line represents the expected correlation.

○ minimal lesion glomerulonephritis, ● membranous glomerulonephritis,
△ amyloid disease, ▲ lupus nephritis, ■ proliferative glomerulonephritis,
□ mixed membranous and proliferative glomerulonephritis.



glomerulonephritis had a better correlation between the dextran and protein values than patients with proliferative glomerulonephritis, in whom the correlation was very poor. It is of interest that the patient (2) with minimal lesion glomerulonephritis had a high selectivity value both during the disease and on recovery, although a slight increase in dextran selectivity occurred on recovery. Only two patients with amyloid disease were studied, and one had a better correlation than the other. The one patient with mixed membranous and proliferative glomerulonephritis had a poor correlation, but the one patient with lupus nephritis had a very good correlation.

Overall the correlation coefficients for Fig. 31 (excluding the minimal proteinuria cases) and Fig. 32 were $r_{16} = + 0.402$ and $r_{16} = + 0.502$ respectively.

3.2. STUDIES ON PROTEIN SELECTIVITY IN RENAL DISEASE

As the protein methods of estimating selectivity correlate so well, the index obtained by the main method in this study, the immunodiffusion method, was used to consider selectivity in relation to various factors. The values for all the patients were considered together in a study of the variation of selectivity values, selectivity in relation to steroid treatment, age, sex, renal function, and total protein excretion. The results of the effect on selectivity of changing plasma protein patterns, by infusion of albumin, are also presented in this section. Finally, selectivity was considered in relation to diagnosis and detailed studies of the ultrastructural glomerular changes.

3.2.1. VARIATION OF SELECTIVITY VALUES

Variation in selectivity values was studied while proteinuria persisted. Changes in selectivity, occurring in both selective and unselective patients, when proteinuria was reduced to trace and normal levels were also studied.

Variations in selectivity over short time intervals

The errors involved in the estimation of selectivity by immunodiffusion were assessed and discussed in the previous chapter (2.3.1). As many of the patients were studied over long time intervals, it was felt important to assess the day to day, or physiological variation, so that the significance of changes in selectivity over longer time intervals of months, or years, could be recognised. The physiological variation in selectivity was therefore estimated from a series of two or more selectivity values, which were determined on samples obtained on different days within one week. The selectivity values in this series ranged from 0.82 - 3.52.

From a total of 150 estimations, on 49 patients, over 69 weeks, it was found that the variation in selectivity values ranged from 0 - 17% with a mean of 5.9%, S.D. $\pm$ 3.8%. A maximum value for the physiological variation in selectivity was therefore taken as $\pm$ 13.5% (mean + 2 S.D.).

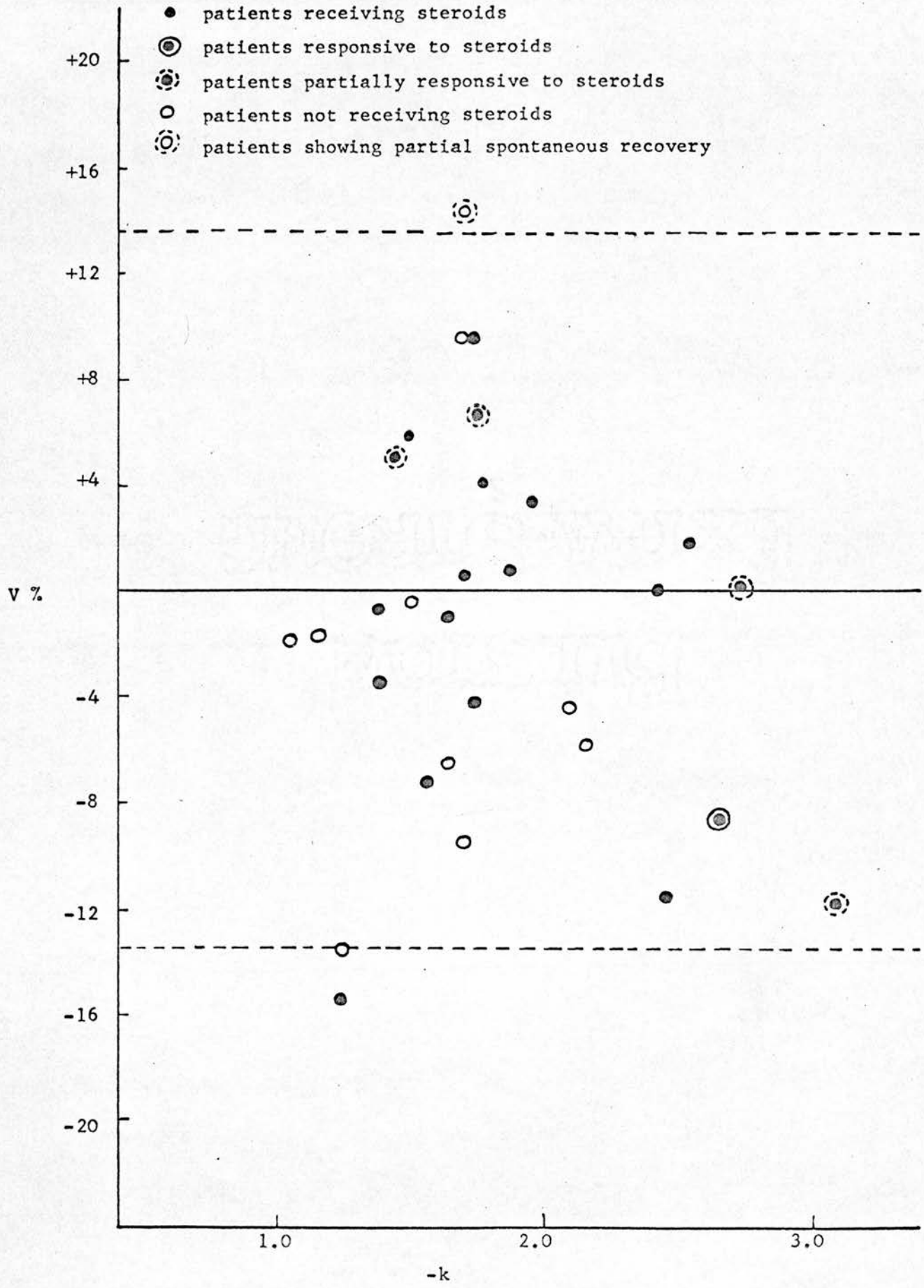
Variation in selectivity over long time intervals during proteinuria

The variation in selectivity values was investigated in 30 patients who were followed up for a minimum period of one year. During the time studied there was no change in treatment. However in some cases there were significant changes in creatinine clearance and protein excretion, particularly in the steroid responsive patients.

Mean selectivity values were calculated over 6 month intervals. The variation was expressed as the % change of the value in the preceeding six

Fig. 33. Variation in selectivity values.

Variation / 6 months / patient (V) plotted against mean selectivity value. The dotted line represents the estimated physiological variation of ± 13.5%.



months, so that one value of variation was obtained from patients studied for 1 year, 2 values from patients studied for 18 months, etc. A total of 77 values of variation were obtained from the 30 patients. The mean value was - 0.3%, range + 55.8 to - 36.5%. The values of over 20% were generally obtained where only one estimation of selectivity was made during a six month period, which suggests that, in part, this represented cumulative physiological and methodological errors, in addition to any genuine change in selectivity. The mean values of variation/patient/six months ranged from + 14.3 to - 15.4%, mean - 1.5%. The majority (93%) of the values were within the estimated physiological variation of $\pm$ 13.5%. Fig. 33 shows the estimations of variations/six month/patient plotted against the mean selectivity value over the time studied. It can be seen that there is no correlation between the direction of variation and selectivity. Eleven patients had a mean increase in selectivity and 17 a mean decrease.

The cumulative variations over periods of over 2 years were studied in 17 patients. The values ranged from + 33.3% to - 19.6%, mean + 2.7% and in only 3 patients was the value over 20%.

The relationship of steroid treatment and variation was also considered. Fig. 33 indicates which patients were receiving steroids and whether they had a complete or partial response (3.2.2). Twenty patients were receiving treatment with steroids, 9 of these had a mean increase in selectivity, and 9 had a mean decrease. Ten patients had no steroid treatment, 2 of these had a mean increase in selectivity and 8 had a mean decrease in selectivity. Expressing these results another way, 37% of the patients had a mean increase in selectivity and of these 82% were receiving steroids. A mean decrease in selectivity was shown by 57% of the patients, and of these 53% were receiving steroids.

TABLE 31.

Changesin selectivity when proteinuria was reduced to trace and normal levels

Selectivity values were estimated pre- and post- steroid treatment and when proteinuria was being abolished, as indicated by the albumin serum:urine ratio (Alb S:U) or the total urinary protein (UP). The relative clearances of the individual proteins are also shown: transferrin (S), γ-globulin (γ), α_2-macroglobulin (α_2) and β-lipoprotein (β).

* Subjects 14 and 15 had no steroid treatment and proteinuria subsided spontaneously.

Subject	Selectivity value			Alb S:U	UP mg./24 hr.	S	γ	α_2	β
	Pre-treatment	Post-treatment	Proteinuria going						
1	2.81	2.73	3.15 2.29	224 320 4480		114 100	18 42	0.06 0.45	
2	2.41	2.47	2.36 2.21	320 2000		83 72	7.7 36	0.32 0.47	
3		2.02	-	5000					
4			1.37	3000	11	120	71	4.6	-
5			- 2.26	3500 640		125	31	0.05	-
6			2.43	256		160	50	0.39	
7	3.01	2.67	2.92	256		80	25	0.09	
8	1.84	2.50	2.85	640		143	13	0.13	
9	2.68	2.59	1.89 1.39 1.71 1.78 -	192 320 470 700 10200	 63	75 125 100 87	19 50 29 50	0.96 4.0 1.6 1.4	
10	2.75	2.67		560	310				
11	2.56	3.25	2.34 1.76 1.30	1160 6000 13700 17900	 17	120 63 62	30 63 62	0.42 1.3 3.8	
12			1.43	6100		85	52	3.5	2.0
13	2.91	3.15		1540 25500	37				
14*		2.26	1.94	448	170	88	25	0.94	-
15*		1.56	1.72 1.28	460 6400		107 143	29 125	1.8 5.7	-

In those patients who were steroid responsive the proteinuria decreased over a period of weeks or sometimes months. During this time, while proteinuria persisted, the variation in selectivity was studied and no significant differences between responsive and non-responsive patients were found. Of the 5 patients who were responding to steroid treatment (Fig. 33), 2 had a mean increase, but 2 had a mean decrease in selectivity. One patient not being treated with steroids, however, had a significant rise in selectivity associated with a partial spontaneous recovery (3.2.2).

Selectivity when proteinuria is reduced to trace and normal levels by steroid treatment

In a few patients with selective proteinuria who responded fully to steroid treatment, it was possible to estimate selectivity values at trace and normal levels of proteinuria. Table 31 gives values of selectivity pre- and post-treatment and when proteinuria had reduced to trace and normal levels. In some cases only the total protein and/or serum:urine albumin level was estimated.

Changes in selectivity values occurred, but at varying values of the serum:urine albumin ratio. In one subject (9) there was a marked reduction in selectivity when the ratio was 320, but in the same patient the change was significantly less when the ratio was 700. In another patient (2) no significant change occurred at a ratio of 2000. However, in most instances significant changes were not detected until the albumin serum:urine ratio was over 400, the criterion of proteinuria for this study.

In 4 patients low indices of selectivity were found at albumin serum:urine levels of 3000 and over; values ranged from 1.30 - 1.43. These were within the range of values found for normal individuals (3.3). In 9 patients the albumin serum:urine levels fell to values ranging from

TABLE 32.

Selectivity values in postural proteinuria

Indices of selectivity (-k) were estimated (a) during proteinuria and (b) when proteinuria was reduced to trace or normal values as indicated by the albumin serum:urine ratio (Alb S:U). The relative clearances of the individual proteins are also shown: transferrin (S), Υ-globulin (Υ), α_2-macroglobulin (α_2) and β-lipoprotein (β).

Subject	-k	Alb S:U	S	Υ	α_2	β
1 a	1.06	26	93	21	7.4	-
b	1.19	640	100	62	5.6	1.8
2 a	1.38	112	58	58	6.5	0.54
b	0.95	5250	110	56	8.5	4.3
3 a	0.75	112	35	30	11	-
b	1.48	768	150	75	3.6	-
4 a	1.26	160	72	50	2.9	1.4
b	1.27	640	72	36	9.3	0.70
5 a	1.01	16	100	57	10	3.0
b	0.73	2130	67	52	15	-

2000 - 25,000; these are also within normal limits (3.3). However, in 2 patients with a long-term response, the urinary protein, although undetectable by the biuret method, was of trace levels as indicated by albumin serum:urine ratios of 640 and 256.

Selectivity when proteinuria is reduced to trace and normal levels spontaneously

Two patients who received no steroid treatment are also included in Table 31. Patient 14 had a focal proliferative glomerulonephritis and Patient 15 had acute proliferative glomerulonephritis. Both fully recovered. Selectivity values in each case showed a reduction when the proteinuria was reduced, with a value of 1.28 for Patient 15, when the albumin serum:urine ratio was 6400. This was within the range of values found in normal individuals (3.3).

Selectivity values were also estimated during proteinuria, and at trace and normal levels of protein excretion, in 5 patients with postural proteinuria. Table 32 gives values of selectivity at two different levels of protein excretion (a and b) for each patient, together with the relative clearances of the individual proteins. There was no significant difference in clearances of any of the proteins between series (a) and (b). β-Lipoprotein was detected in 3 of the (a) and 3 of the (b) samples. Albumin serum:urine ratios and selectivity values during proteinuria (a) ranged from 16 - 160 and 0.75 - 1.38 respectively. When proteinuria was reduced to trace or normal levels, values ranged from 640 - 5250 and 0.73 - 1.48 respectively. Mean selectivity values for (a) and (b) were 1.09 and 1.12 respectively.

The selectivity of the proteinuria in acute ischaemic renal failure was repeatedly estimated from the onset of the illness to death or recovery

TABLE 33.

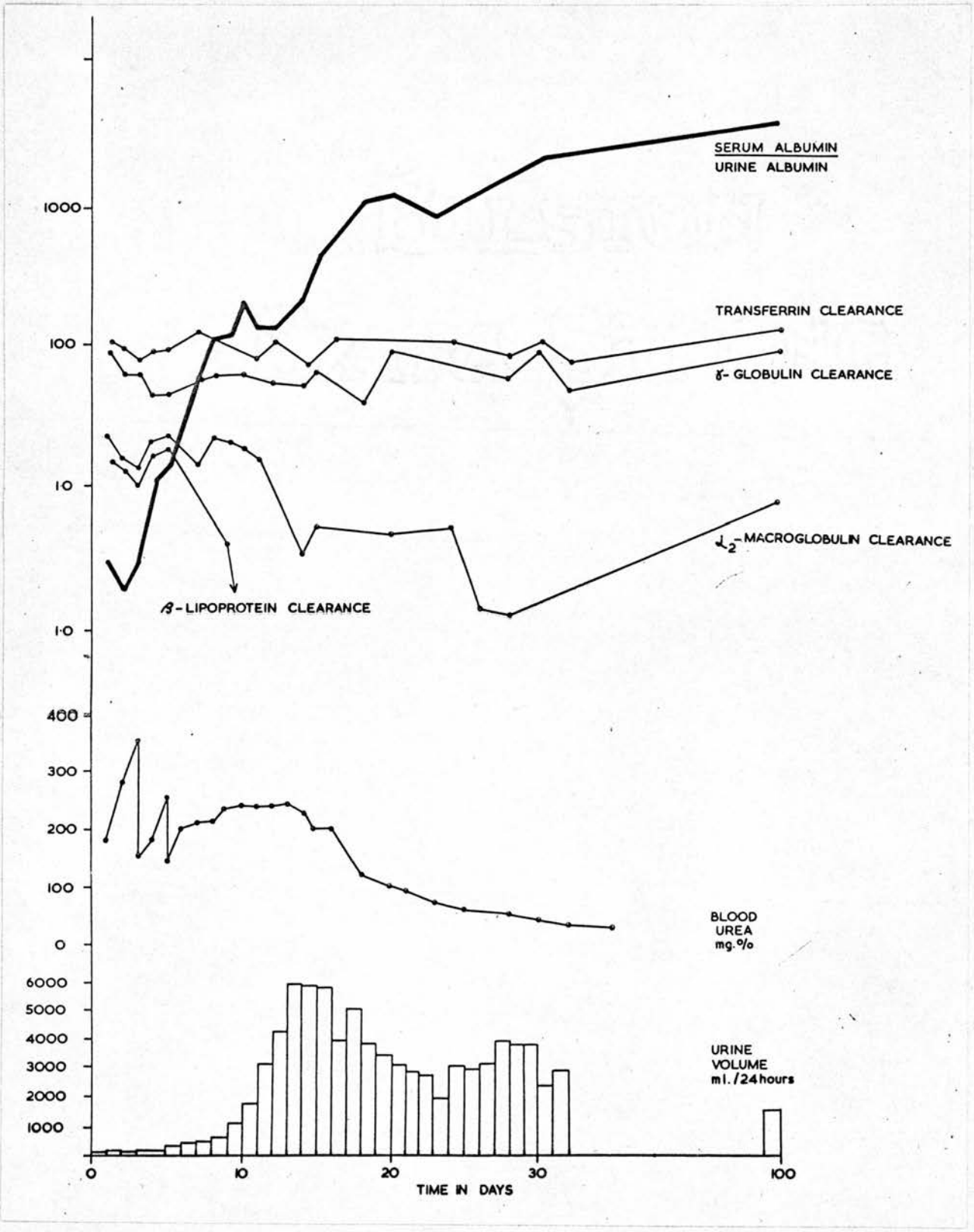
Selectivity values in acute ischaemic renal failure

Indices of selectivity (-k) were estimated at 3 degrees of proteinuria as indicated by the albumin serum:urine ratios (Alb S:U) of (a) < 200, (b) 200 - 1000, (c) > 1000. The relative clearances of the individual proteins are also shown: transferrin (S), Υ-globulin (Υ), α_2-macroglobulin (α_2) and β-lipoprotein (β).

Subject	Alb S:U	S	Υ	α_2	β	No. of determinations	-k	Blood urea mg./100ml.
1 a	68	96	54	20	9.6	12	0.66	223
b	520	80	59	3.1	<0.5	3	1.44	115
c	2250	108	76	3.5	<1.0	4	1.45	35
2 a	28	54	40	5.6	0.8	3	1.10	218
b	460	80	58	14	<0.5	3	0.79	186
c	2340	121	69	6.8	<1.0	5	1.17	38
3 a	72	75	32	10	1.3	2	1.13	214
b	400	86	74	15	4.9	2	0.87	42
c	1790	98	68	15	4.4	5	0.89	48
4 a	100	82	60	18	5.1	3	0.80	241
b	600	83	60	17	1.5	6	1.08	216
c	1540	118	68	15	5.3	5	0.86	94
5 a	130	89	50	34	2.2	6	0.40	247
b	450	121	59	15	<1.0	3	0.82	110
6 a	96	69	49	12	1.5	2	1.08	214
b	380	118	61	27	4.3	3	0.84	211

Fig. 34. Selectivity during the course of the illness in acute ischaemic renal failure.

The serum:urine albumin ratio showed a marked rise, while the blood urea fell. The urine volume rose to reach a maximum in the diuretic phase. Clearances of the individual proteins showed no systematic change, and the values corresponded to an unselective proteinuria.



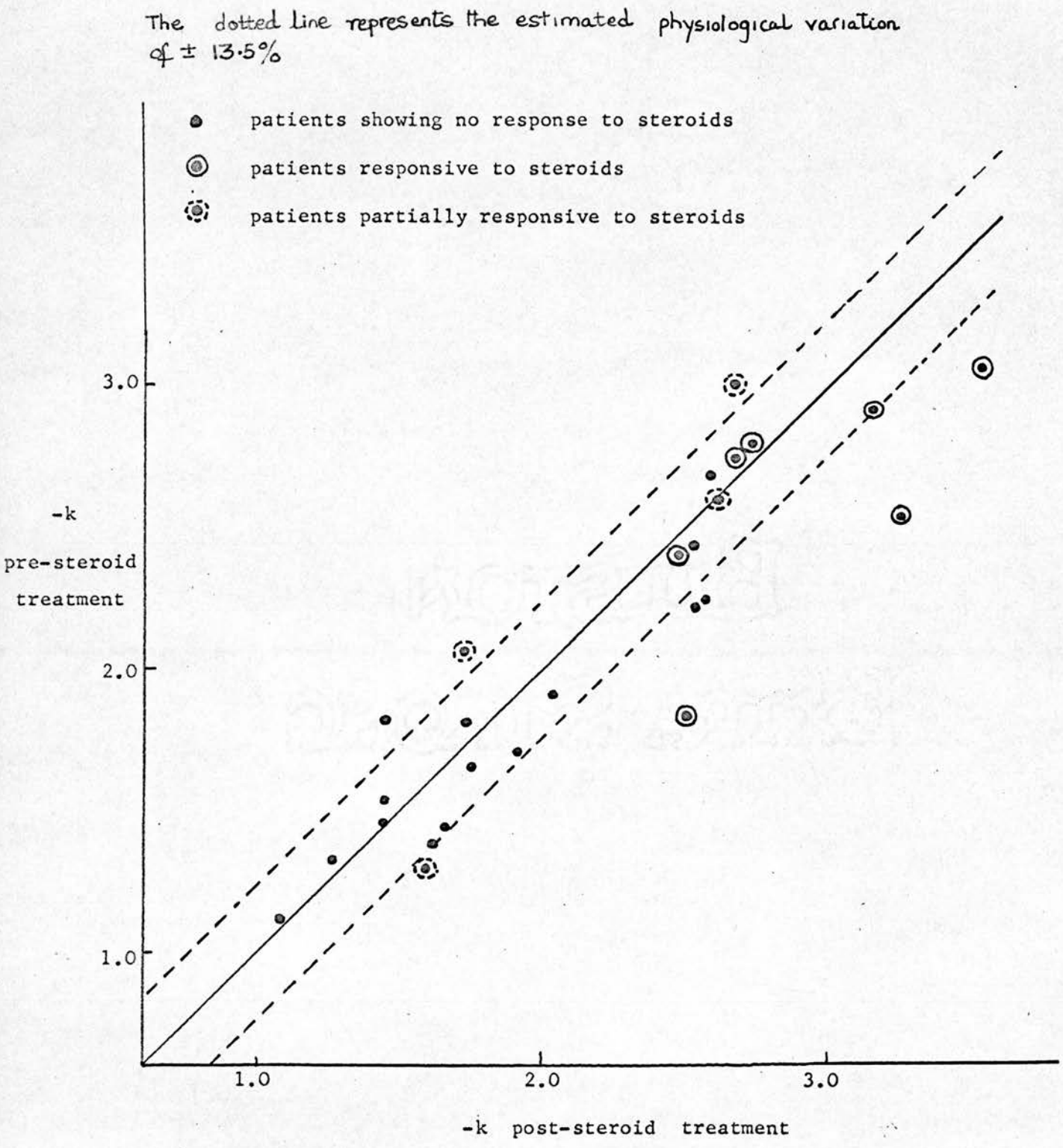
in 6 patients. Table 33 gives the selectivity values for each patient at 3 different levels of protein excretion, as defined by albumin serum:urine ratios of (1) < 200, (2) 200 - 1000, and (3) > 1000. The number of determinations and the blood urea are also given. Clearances of γ-globulin rose slightly as the proteinuria reduced to normal values. β-Lipoprotein was always detected in the early phases of the illness, but in three cases it was not detected when the urinary protein concentration fell on recovery. Values of selectivity did not change systematically or significantly with recovery and the return of the degree of proteinuria towards normal values (3.3). Mean values and ranges for the different degrees of proteinuria were (1) < 200, $-k = 0.86$, range = 0.40 - 1.13; (2) 200 - 1000, $-k = 0.97$, range = 0.79 - 1.44; (3) > 1000, $-k = 1.09$, range = 0.86 - 1.45. Fig. 34 shows the individual protein clearances, serum:urine albumin ratio, blood urea and urine volume for patient 1 during the course of the illness.

Summary

The variation in selectivity values within one week was estimated to have a maximum value of $\pm$ 13.5%. Variation over longer time intervals of one or two years, whether or not the patient was being treated with steroids and while proteinuria persisted, was not found to be significantly different from the value of $\pm$ 13.5%. When proteinuria was reduced, however, to trace or normal levels by steroid treatment or spontaneously, the index of selectivity fell, from the highly selective values associated with the ability to abolish proteinuria (3.2.2), to values approaching those of normal subjects. The changes became significant at varying levels of protein excretion between albumin serum:urine ratios of 200 and 2000. Selectivity values in postural proteinuria and acute ischaemic renal failure, however, did not significantly change as the proteinuria returned

to normal levels. In these patients the proteinuria remained unselective at both abnormal and normal levels of protein excretion.

Fig. 35. Selectivity values pre- and post-steroid treatment.



3.2.2. SELECTIVITY AND STEROID TREATMENT

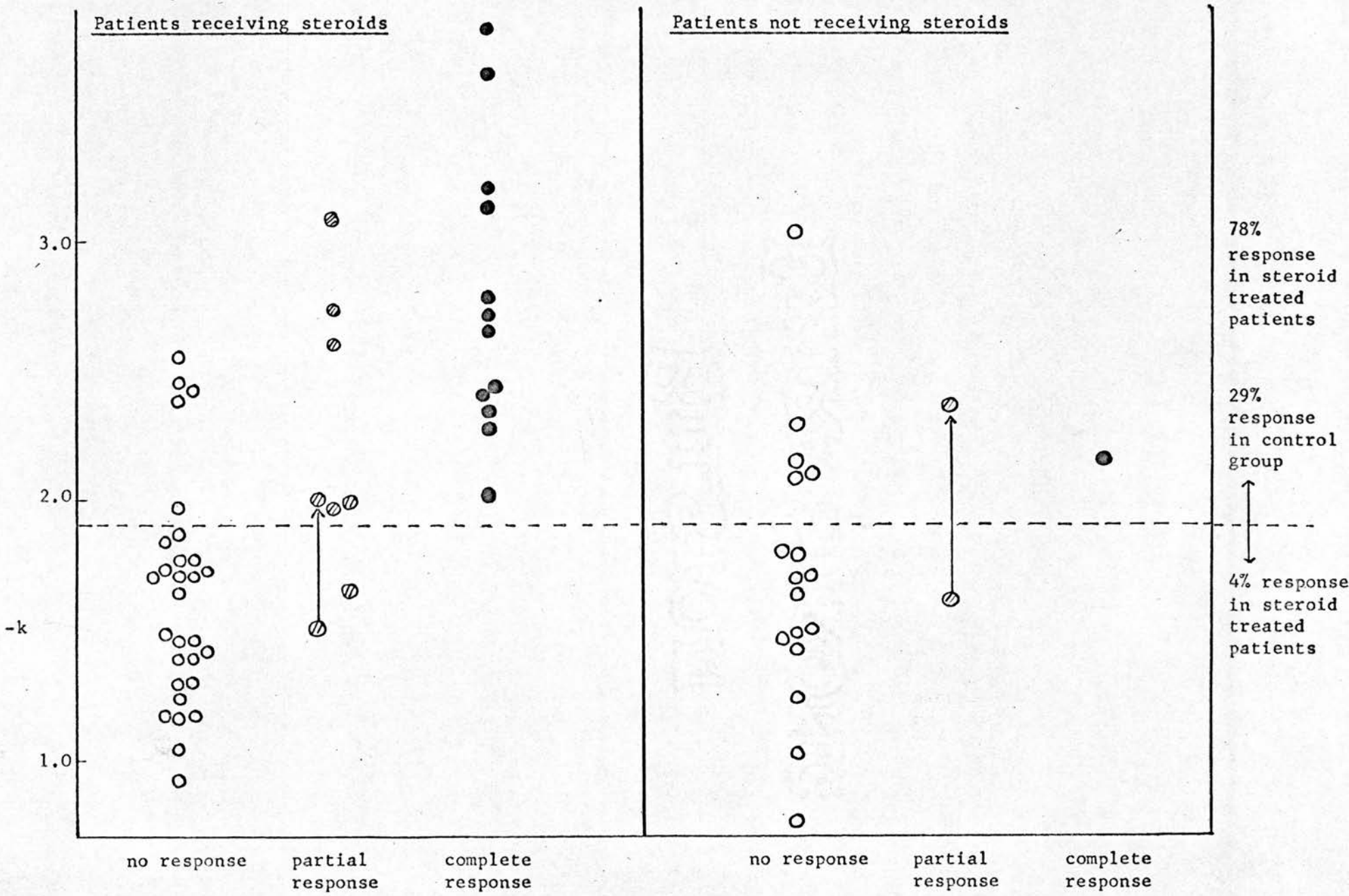
A proportion of the patients received steroid treatment and this group included patients with almost every type of renal disease in this study, excluding amyloid disease, postural proteinuria and acute ischaemic renal failure. The effect on selectivity values of commencing steroid treatment, and the relationship of selectivity values and response were considered. Results of studies of variations in selectivity, while the patient was receiving steroids and when proteinuria diminished to trace and normal values, as a result of steroid treatment, have already been presented.

Effect of commencing steroid treatment on selectivity values

Indices of selectivity were estimated before and after steroid treatment in 26 patients. In almost every case two or three values were estimated, over the weeks during which the patient was investigated and assessed, before commencing steroid treatment. A minimum of 2 estimations of selectivity, after steroid treatment was commenced, was made over the following 6 months, or while proteinuria persisted, whichever was shorter. Since selectivity values did not change significantly while the patient was receiving steroids and proteinuria persisted, a mean value of selectivity over the period studied was taken for each patient. Fig. 35 shows mean pre- and post-steroid selectivity values plotted against each other. The majority of values showed no significant change, and were within, or almost within, the estimated physiological variation. This was true of most of the patients who responded to the treatment, as well as those who did not respond. (Criteria of response are outlined below). However, in three patients, who fully responded to steroids, there was a marked rise in selectivity values after treatment.

Fig. 36. Correlation of selectivity values with responsiveness to steroids.

Each dot represents the mean selectivity value of one patient. Two patients showed a significant rise in selectivity which is indicated by the linked dots.



Correlation of selectivity values and steroid response

The relationship of selectivity values and steroid responsiveness was investigated in 48 patients. Each patient was studied during steroid treatment for three months, or while proteinuria persisted, whichever was shorter. A control group of 19 patients, who were not treated with steroids, was also studied for a minimum period of three months for each patient. Since selectivity values did not change significantly while the patient was receiving steroids and proteinuria persisted, a mean value of selectivity during the period studied was taken for each patient. A complete response was defined by abolition of proteinuria, with the urinary protein concentration falling to less than 30 mg.% and a rise to normal values in the creatinine clearance, if it was reduced. Partial response was defined by a reduction in proteinuria by 70% and a rise in the creatinine clearance, if reduced, to normal values, or to a value at least 50% higher than the original value. Patients who did not fall into either of these categories were considered as having no response. Fig. 36 shows selectivity values plotted against response for the patients receiving steroids and the control group. The results are very striking. Of the 48 patients receiving steroids, 19 (40%) had a complete or partial response and of these, 18 (95%) had selectivity values of over 1.9. Seven patients (15%) had a partial response, of these 6 (80%) had selectivity values of over 1.9 and 12 (25%) had a complete response, of these 12 (100%) had selectivity values of over 2.0. Of the whole group of steroid treated patients, 23 patients (48%) had selectivity values of over 1.9 and of these 18 (78%) partially or wholly responded. Twenty-five patients (52%) had selectivity values of under 1.9, and of these 1 (4%) partially responded. In the control group 2 patients (11%) responded, and both had selectivity

values of over 2.1. Of the 7 control patients with selectivity values of over 1.9, 2 (29%) responded.

Although patients with minimal lesion glomerulonephritis had the most selective type of proteinuria (3.2.4), the selective steroid-responsive patients were not confined to this disease group. One patient with proliferative glomerulonephritis and 2 patients with lupus nephritis fully responded. One patient with proliferative glomerulonephritis, 1 patient with lupus nephritis and 1 patient with mixed membranous and proliferative glomerulonephritis partially responded. In the control group the patient who fully responded and the patient who partially responded both had proliferative glomerulonephritis.

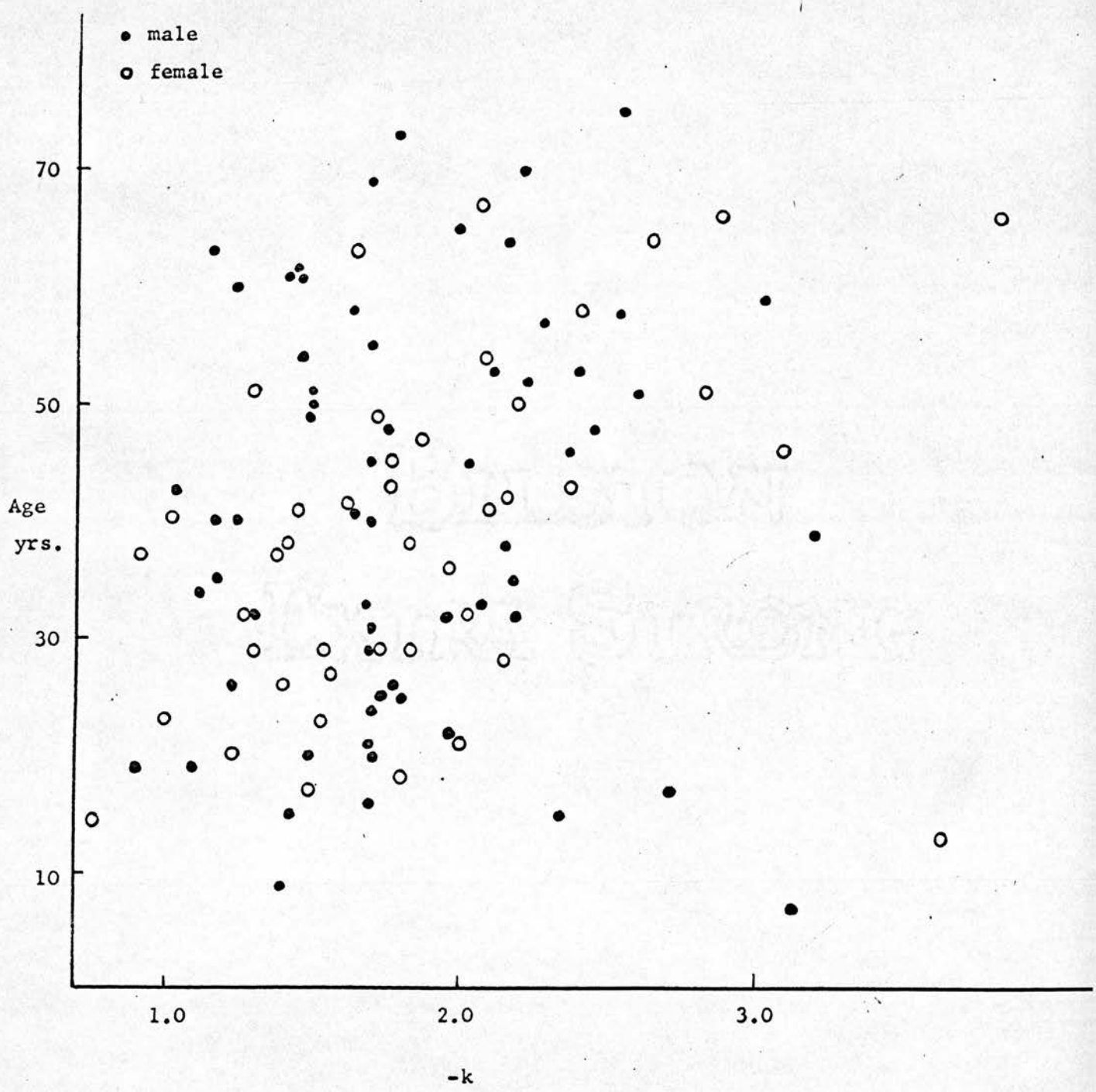
In both the steroid treated and control groups there was a large overall rise in selectivity in a patient who had a partial response. The control patient had a mean positive variation in selectivity/six months of 14.3% over 18 months, and the steroid treated patient had a mean positive variation in selectivity/six months of 6.7% over 18 months. The increases in selectivity to values of over 2.0 shown in Fig. 36 were due to an accumulation of the positive variations.

Summary

Selectivity values showed no significant change when steroid treatment was commenced and while proteinuria persisted, with the exception of three responsive patients, in whom the selectivity rose. Responsiveness to steroid treatment and spontaneously subsiding proteinuria both correlated extremely well with selectivity. There was a high recovery rate at selectivity values of over 1.9. Recovery was very much more frequent in the steroid treatment group.

Fig. 37. Correlation of selectivity values with age and sex.

Each dot represents the mean selectivity value of one patient.



3.2.3. SELECTIVITY IN RELATION TO OTHER PARAMETERS

Mean values for the whole group of patients were considered together in relation to age, sex, renal function, and total protein excretion. Results of selectivity studies following infusions of albumin are also given here.

Age and Sex

Mean indices of selectivity were compared to the age of the patient in 107 cases. This group included those patients with histological evidence of glomerular disease, but excluded patients with postural proteinuria and acute ischaemic renal failure.

Fig. 37 shows the selectivity values plotted against age. Although some of the children had a selective proteinuria, there was an obvious positive correlation, with a tendency for the more selective proteinuria to be associated with the older patient. The correlation coefficient $r_{107} = + 0.549$, $p < 0.001$.

Of the 130 patients studied, 56 were female and 74 were male. No significant difference in selectivity values was found. For females the mean value was 1.79, S.D. $\pm$ 0.67, coefficient of variation 37%, standard error of the mean 3.9%, and for males the mean value was 1.72, S.D. $\pm$ 0.57, coefficient of variation 33%, standard error of the mean 5.2%. Fig. 37 also shows the sex of the patient in relation to selectivity.

Total protein excretion

Mean indices of selectivity were compared to the total protein excretion in 100 patients. In every case the urinary protein concentration was over 30 mg.% and only patients with histological evidence of glomerular disease were included, those with postural proteinuria and acute ischaemic renal failure being excluded.

Fig. 38. Correlation of selectivity values with total protein excretion.

Each dot represents the mean selectivity value and the mean value of total protein excretion of one patient. UP = total protein excretion in g./24 hr.

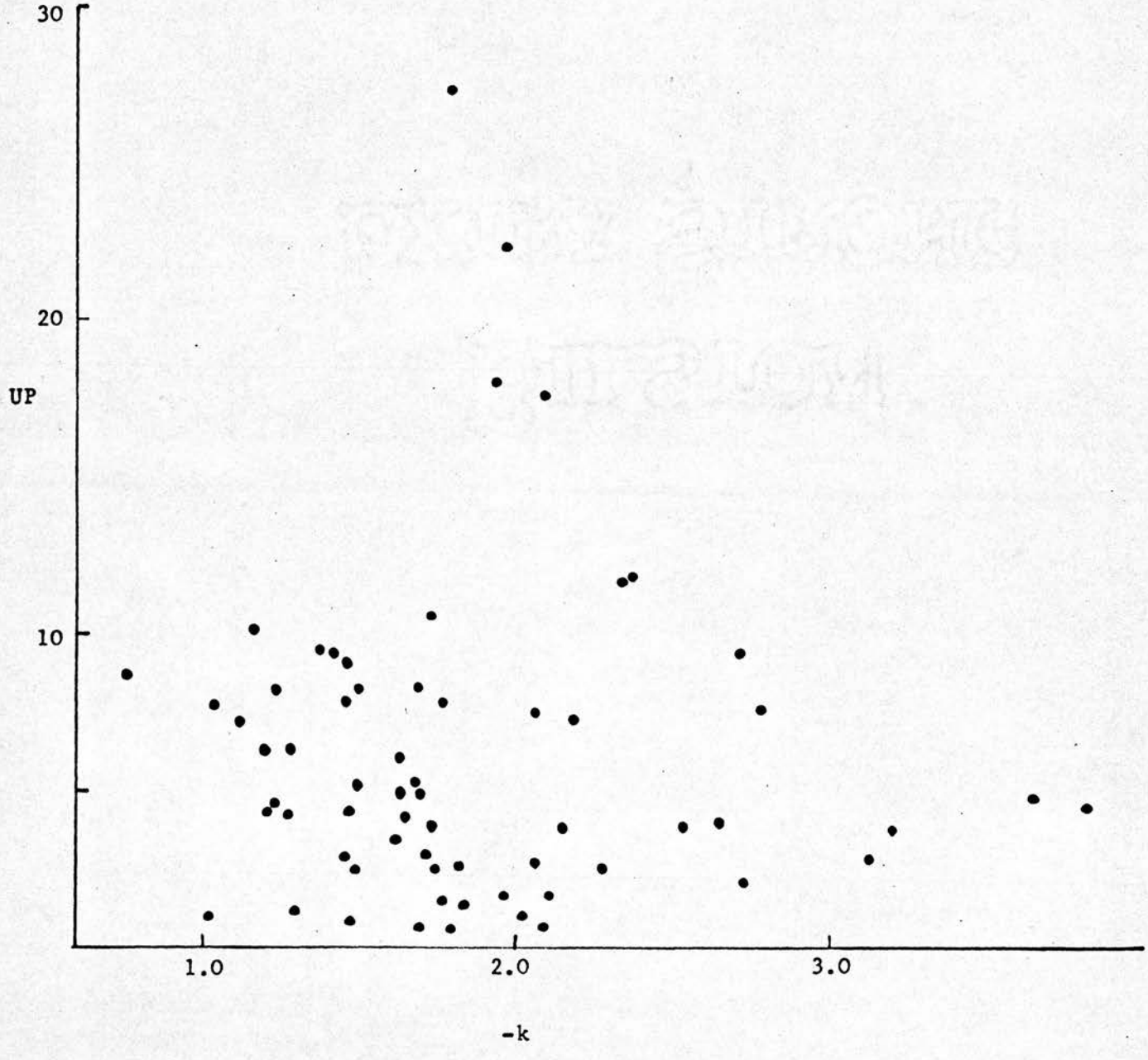


Fig. 39. Correlation of selectivity values with creatinine clearance.

Each dot represents the mean selectivity value and the mean value of creatinine clearance of one patient. Cr Cl = creatinine clearance in ml./min. Correlation coefficient $r_{los} = +0.303$, $p < 0.01$.

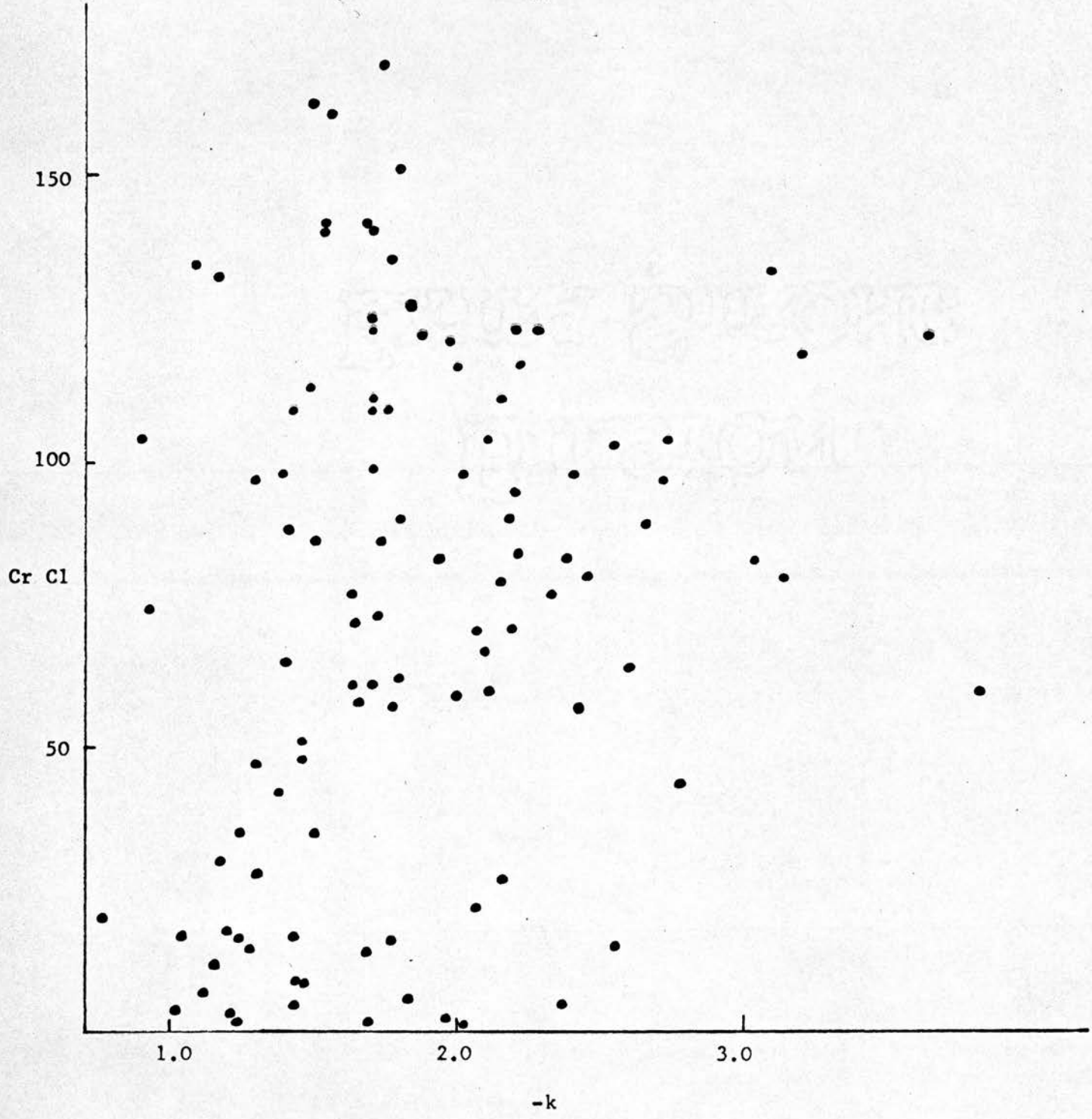


Fig. 38 shows mean selectivity values plotted against mean total protein excretion. There is no obvious correlation and the correlation coefficient $r_{100} = +0.239$, $p < 0.05$. When values for the different types of renal diseases were studied there was still no obvious correlation. Selective patients with minimal lesion glomerulonephritis often excreted as much, or more, protein as an unselective patient with any other type of renal disease.

Renal function

Mean indices of selectivity were compared to the glomerular filtration rate, as measured by the creatinine clearance, in 105 patients. This group included those patients with histological evidence of glomerular disease, but excluded patients with postural proteinuria and acute ischaemic renal failure.

Fig. 39 shows the selectivity values plotted against mean values of creatinine clearance. There was a degree of positive correlation, with a tendency for good renal function to be associated with high selectivity. However there was a group of patients with selectivity values of the order of 2,0 with very low creatinine clearances of under 30 ml./min. Patients in this category had either proliferative glomerulonephritis, or amyloid disease. Amongst the patients with selectivity values under 1.6 there was a substantial proportion with creatinine clearances of over 80 ml./min. This group of patients often had proliferative glomerulonephritis and one case had exercise haematuria.

This indicated that some types of renal disease had a closer correlation between selectivity values and renal function than others. Patients with minimal lesion glomerulonephritis all had good renal function associated with their high selectivity values, and patients with chronic

TABLE 34.

Albumin infusion experiments

Values of selectivity (-k) were estimated before, during and after infusion of albumin. Details are given of the samples taken, urine protein (UP), creatinine clearance (Cr Cl), serum protein, albumin serum:urine ratio (Alb S:U) and the relative clearances of the individual proteins: transferrin (S), Υ-globulin (Υ), α_2-macroglobulin and β-lipoprotein (β).

Subject	Infusion	Serum samples	Urine samples	Urine vol.	UP		Cr Cl	Serum protein		Alb S:U	S	Υ	α_2	β	-k
					mg.%	mg/min.	ml./min	Total	% Alb						
1	Mean pre-infusion value														0.87
	Control	10.30	10.30-10.30	2500			26	7.2	51	512	125	83	16	-	0.82
	100 g Albumin 10.30-11.00														
		11.00	11.00-11.40	160			29	7.8	65	224	100	50	3.9	1.4	1.27
		11.40						7.8	65						
		12.00	11.40-12.20	220			35			320	120	43	3.2	1.2	1.34
		12.40	12.20-1.00	190			32	7.8	55	448	100	50	9.3	4.0	0.94
		4.30	1.00 - 8.00	1150			27	7.7	54	512	88	58	13	-	0.84
2	Mean pre-infusion values						30								2.16
	Control		7.00-2.30	512	1350	4.55	25			1.5	150	30	0.41	0.07	2.21
		2.30						3.9	29						
	50 g Albumin 2.30-4.30		2.30-3.30	31	2300	11.9	41			1.25	125	36	0.87	0.33	1.77
		3.30						4.2	31						
			3.30-4.30	26	1650	7.15	33			1.5	150	37	0.50	0.21	1.96
		4.30						4.3	41						

	Frusemide given		4.30-5.30	140	650	15.2	21			3	30	19	0.20	0.09	2.01
		5.30						4.1	53						
3	Mean pre-infusion values						11								1.24
	Control		7.00-3.00	475	340	3.37	15			12	100	60	4.5	1.7	1.23
		2.40						4.0	43						
	50 g Albumin 3.00-5.00		3.00-5.00	83	400	2.77	15			12	120	60	4.0	2.0	1.23
		5.00						4.0	48						
			5.00-6.40	79	320	2.55	18			12	85	50	4.0	1.5	1.23
		6.40						3.9	59						
			6.40-8.40	83	360	208	13			14	140	50	4.6	1.6	1.27
		8.40						4.4	43						

renal failure all had poor renal function associated with their low selectivity values. Patients with membranous glomerulonephritis and diabetic glomerulosclerosis also had a good correlation between selectivity and renal function, with the higher selectivity values generally being associated with good renal function and vice versa. However patients with proliferative glomerulonephritis had a very poor correlation, with some high selectivity values being associated with poor renal function and vice versa. Patients with amyloid disease also had little correlation, renal function generally being poor, but selectivity values high.

Selectivity on infusion of albumin

Selectivity values were estimated in three patients, before, during and after infusion of serum albumin. One patient (1) was in the diuretic phase of acute ischaemic renal failure and two had significant glomerular disease. Of the latter, one had lupus nephritis (2) and a selective proteinuria, the other had proliferative glomerulonephritis (3) and an unselective proteinuria.

Results are shown in Table 34 which also gives details of the urine and serum samples taken, urine protein concentrations, creatinine clearance and serum proteins. In every subject the serum albumin concentration rose by at least 12%. In subject 1 both the urine protein concentration, as indicated by the albumin serum:urine ratio, and the creatinine clearance also rose. The selectivity value rose by 63%, from 0.82 to 1.34. The maximum values for serum albumin, urine protein concentration, creatinine clearance and selectivity all occurred at the same time, at the beginning of, or during, the second urine collection period.

In subject 2 the urine protein concentration and creatinine clearance rose, but the selectivity value fell by 20% from 2.21 to 1.77.

Maximum values of urine protein concentration and creatinine clearance coincided with the minimum selectivity value, although the maximum serum albumin concentration occurred slightly later.

In subject 3 the creatinine clearance rose, but the urine protein concentration fell slightly from the control value. However, the urinary protein concentration was higher during the first collection period after commencing the infusion, than the last collection period. There was no significant change in selectivity values.

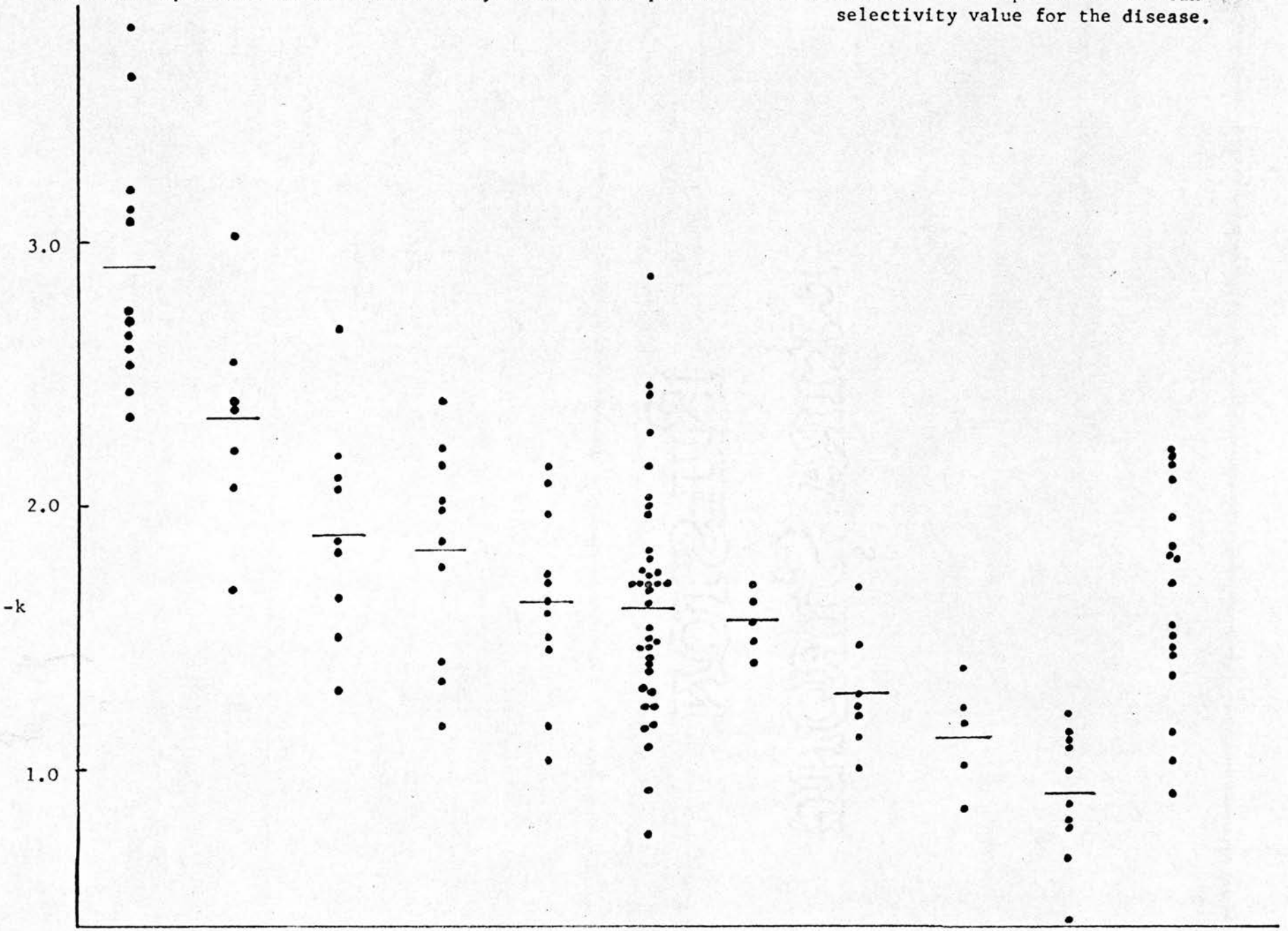
TABLE 35.

Protein selectivity in relation to diagnosis

Diagnosis	No. of cases	Mean selectivity	Range of values
Minimal lesion glomerulonephritis	12	2.91	2.34 - 3.82
Amyloid disease	7	2.33	1.68 - 3.03
Diabetic glomerulosclerosis	9	1.89	1.30 - 2.68
Lupus nephritis	10	1.84	1.17 - 2.40
Membranous glomerulonephritis	11	1.64	1.04 - 2.15
Proliferative glomerulonephritis	37	1.62	0.76 - 2.78
Mixed membranous and proliferative glomerulonephritis	5	1.56	1.40 - 1.70
Chronic renal failure	7	1.28	1.00 - 1.69
Postural proteinuria	5	1.11	0.75 - 1.38
Acute ischaemic renal failure	10	0.90	0.40 - 1.20
Miscellaneous: Toxaemia of pregnancy	3	1.33	1.13 - 1.50
K depleted hypertensives	3	2.18	2.16 - 2.20
Post-partum renal disease	2	1.43	1.02 - 1.83
Renal vein thrombosis	2	1.87	1.79 - 1.95
Exercise haematuria	1	0.90	
Pyelonephritis	1	1.43	
Multiple myeloma	1	1.44	
Proteinuria of unknown origin	4	1.79	1.54 - 2.10
Preliminary series in pregnancy - no biopsy			
Toxaemia	7	1.69	0.86 - 3.15
Accidental haemorrhage	7	1.14	0.82 - 1.65

Fig. 40. Correlation of selectivity values with diagnosis.

Each dot represents the mean selectivity value of one patient. The horizontal line represents the mean selectivity value for the disease.



3.2.4. SELECTIVITY IN RELATION TO DIAGNOSIS

Selectivity values were compared with the specific diagnosis in all of the 130 patients. The selectivity is considered here simply in relation to the type of renal disease; selectivity in relation to the degree and extent of the ultrastructural glomerular lesion will be considered later (3.2.5). The variation in selectivity values over 1 year periods was found to be within the physiological variation and in patients who were studied over longer time intervals of 2 years the cumulative variation was not generally greater than 20%. In addition, when steroid treatment was commenced there was generally no significant change in selectivity values for as long as proteinuria persisted. Therefore it was considered valid to take a mean value of selectivity for each patient.

Ranges and mean values of selectivity for the different types of renal diseases studied are given in Table 35, and Fig. 40 shows the data diagrammatically. Most of the different types of renal disease had a wide range of selectivity values, although the mean group values showed significant differences and ranged from 0.90 - 2.91. Patients with minimal lesion glomerulonephritis had by far the most selective proteinuria of all, although some patients with amyloid disease, proliferative glomerulonephritis, lupus nephritis and diabetic glomerulosclerosis had selectivity values within the minimal lesion range. Patients with amyloid disease had, with one exception, selectivity values of over 2.0. Patients with lupus nephritis and diabetes both had a range of selectivity values, but the mean values were above the mean for all the patients studied (1.74). Proteinuria in proliferative glomerulonephritis had an enormous range of selectivity values, overlapping with all the different disease groups, from the least to the most selective.

Patients with mixed membranous and proliferative glomerulonephritis, and membranous glomerulonephritis also had a range of values with group means which were slightly more unselective than the overall mean of 1.74. The values in membranous glomerulonephritis were quite different from those in minimal lesion glomerulonephritis. Patients with chronic renal failure, postural proteinuria and acute ischaemic renal failure all had very unselective proteinuria, particularly those with acute ischaemic renal failure.

In the miscellaneous group of patients, those with potassium depletion and hypertension were found to have a selective proteinuria, those with multiple myeloma, pyelonephritis, and exercise haematuria to have an unselective proteinuria, and those with proteinuria of unknown origin or renal vein thrombosis to have proteinuria of intermediate selectivity. Two patients with post-partum renal disease were found to have different selectivity values, one intermediate and one unselective.

The preliminary study of proteinuria in pregnancy indicated that the proteinuria of toxaemia was more selective than the proteinuria following accidental haemorrhage. However, the 3 patients with toxaemia, included in the miscellaneous group, who all had a biopsy, had a less selective proteinuria than those of the initial series.

Although each type of renal disease had a range of selectivity values the patterns were sometimes very different - for example, minimal lesion glomerulonephritis had a completely different range from that of mixed membranous and proliferative glomerulonephritis, membranous glomerulonephritis, chronic renal failure, postural proteinuria and acute ischaemic renal failure. Postural proteinuria and acute ischaemic renal failure also had a quite different range of selectivity values from that of amyloid disease. Student's t tests were carried out to establish the significance of these

TABLE 36.

Student's t tests on selectivity values in different renal diseases

t Tests were carried out to establish the significance of the different selectivity values found in the following renal diseases: minimal lesion glomerulonephritis (Min), amyloid disease (Am), diabetic glomerulosclerosis (D), lupus nephritis (L), membranous glomerulonephritis (M), proliferative glomerulonephritis (Pr), mixed membranous and proliferative glomerulonephritis (Mix), chronic renal failure (C), postural proteinuria (P) and acute ischaemic renal failure (Ac).

(a) Values of t when selectivity values in two renal diseases were compared are shown in the bottom left-hand half of the table and the significance (p) is shown in the top right-hand half.

	Min	Am	D	L	M	Pr	Mix	C	P	Ac
Min		0.01	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
Am	2.82		0.025	0.0125	0.0025	0.0005	0.0025	0.0005	0.0005	0.0005
D	5.21	2.15		0.80	0.10	0.05	0.10	0.0025	0.0025	0.0005
L	5.94	2.55	0.27		0.15	0.10	0.10	0.0025	0.0025	0.0005
M	7.41	3.79	1.44	1.23		0.90	0.70	0.025	0.005	0.0005
Pr	9.00	4.08	1.72	1.48	0.14		0.80	0.025	0.01	0.0005
Mix	9.26	4.20	1.74	1.59	0.48	0.31		0.025	0.0025	0.0005
C	8.73	5.86	3.43	3.36	2.28	2.03	2.33		0.30	0.0025
P	8.42	6.11	3.87	3.89	2.98	2.61	4.16	1.23		0.10
Ac	12.60	9.15	6.43	6.51	5.39	5.09	5.71	3.58	1.58	

t

(b) Summary of the significance of the differences in selectivity values.

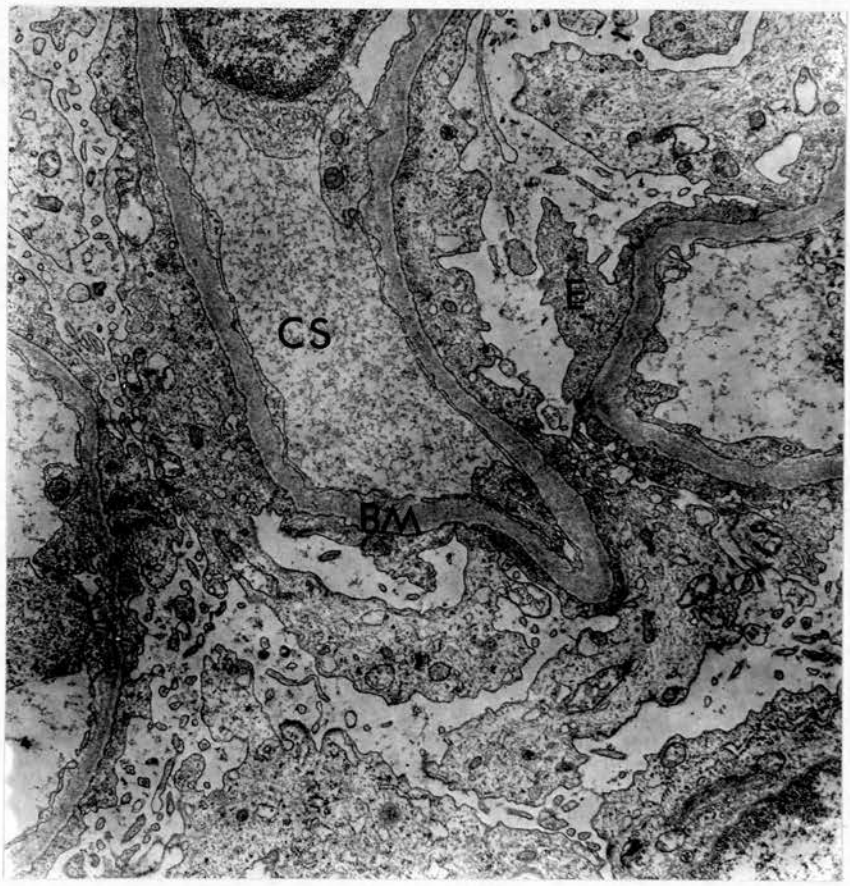
Disease	Significantly different from	Probably significantly different from	Not significantly different from
Min	D,L,M,Pr,Mix,C,P,Ac	Am	-
Am	M,Pr,Mix,C,P,Ac	Min, D, L	-
D	Min, C, P, Ac	Am	L, M, Pr, Mix
L	Min, C, P, Ac	Am	D, M, Pr, Mix
M	Min, Am, P, Ac	C	D, L, Pr, Mix
Pr	Min, Am, Ac	C, P	D, L, M, Mix
Mix	Min, Am, P, Ac	C	D, L, M, Pr
C	Min, Am, D, L, Ac	M, Pr, Mix	P
P	Min, Am, D, L, M, Mix	Pr	Ac
Ac	Min,Am, D,L,M,Pr,Mix,C	-	P

differences. The results are shown in Table 36a and summarised in Table 36 b. Selectivity values in minimal lesion glomerulonephritis, amyloid disease, chronic renal failure, postural proteinuria, and acute ischaemic renal failure were all statistically significantly different from those of other diseases, but values in membranous glomerulonephritis, diabetic glomerulosclerosis, proliferative glomerulonephritis, and mixed membranous and proliferative glomerulonephritis were not significantly different from one another.

Fig. 41. Minimal lesion glomerulonephritis

In most cases the basement membrane remains normal. There is complete loss of epithelial cell pedicels.

Magnification x 9,600.



The following abbreviations are used in the above and in all subsequent electron microscopic photographs:-

BM	basement membrane
E	epithelial cell
CS	capillary space
EN	endothelial cell
P	pedicels
D	deposit
W	white blood cells

3.2.5. SELECTIVITY AND GLOMERULAR ULTRASTRUCTURE

Selectivity and glomerular ultrastructure were studied in 119 patients. Renal biopsy tissue from the majority of the patients was examined by electron, as well as light microscopy. Selectivity values for the different types of renal disease have already been considered (Fig. 40 and Table 35). In this section a brief description of the microscopic findings is given for each type of renal disease, and, where possible, the degree of damage is correlated with individual selectivity values.

Minimal lesion glomerulonephritis

Selectivity values ranged from 2.34 - 3.82, mean 2.91.

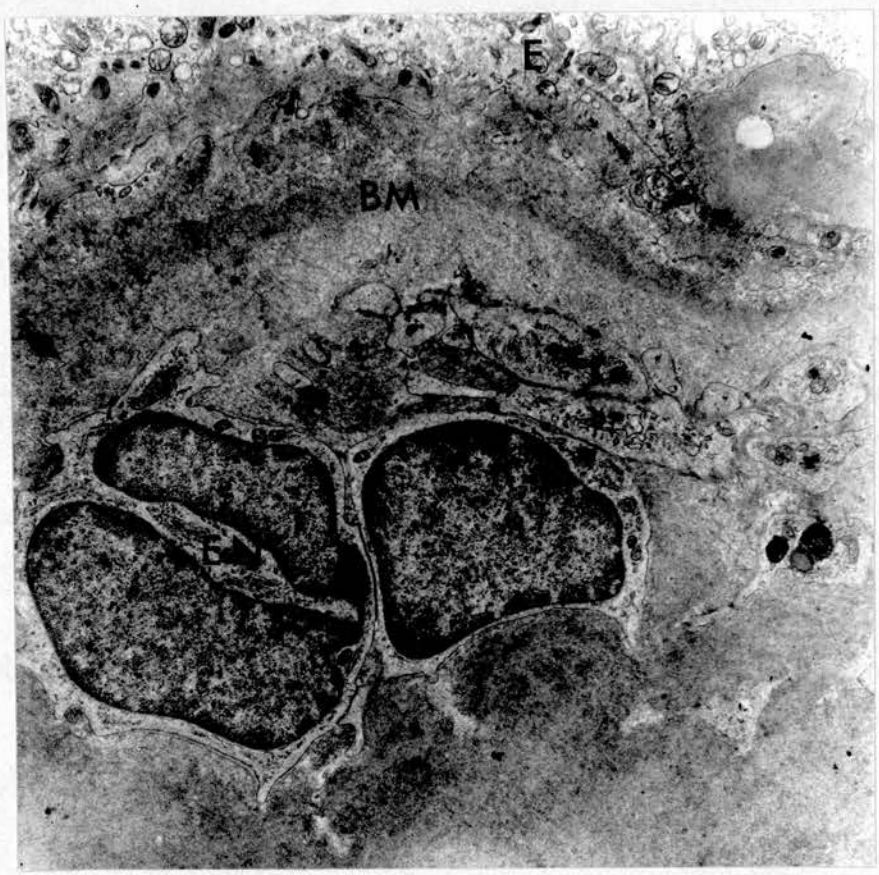
In general, biopsies in these individuals showed no significant light microscopy lesion, although occasionally a very slight degree of endothelial cell proliferation was noted. Electron microscopy of the glomeruli showed the same basic changes (Fig. 41). A minor degree of endothelial cell proliferation was noted in some cases. The epithelium showed the most marked changes, with loss of pedicel structure producing a continuous layer of epithelial cytoplasm over the external aspect of the basement membrane. Folli bodies and localised ballooning of epithelial cytoplasm were also seen. The basement membrane was relatively normal, although a focal folding and tortuosity was sometimes noticed. In two patients a definite slight increase in basement membrane thickness was noted, when the initial biopsy specimen was compared to a biopsy specimen taken some months later. In these two patients selectivity values were 3.20 and 2.54. Where biopsies were available before and after treatment, steroid therapy resulted in a reappearance of normal epithelial pedicel structure.

With the exception of the patients with the thickened basement membrane, glomerular changes in all the patients were very similar and

Fig. 42. Amyloid disease.

Epithelial pedicel structure is lost. The basement membrane is grossly distorted by deposition of amyloid material. At higher magnification this has a distinctive fibrillar appearance.

Magnification x 7,000.



therefore no comparison was made of the individual glomerular changes with individual selectivity values.

Amyloid disease

Selectivity values ranged from 1.68 - 3.03, mean 2.33.

Glomeruli affected by amyloidosis showed a homogeneous deposition of hyaline material, initially in the region of the basement membrane. This had progressed in some instances to obliteration of the capillary loops. On electron microscopy (Fig. 42) the appearances were entirely characteristic and showed the amyloid deposits had a distinctly fibrillar structure, and thickened the basement membrane in an irregular fashion.

The patient who had by far the lowest selectivity value of 1.68 had no renal biopsy, so unfortunately in this case it was impossible to compare the glomerular appearances with those of all the other patients with much higher selectivity values.

Diabetic glomerulosclerosis

Selectivity values ranged from 1.30 - 2.68, mean 1.89.

On light microscopy the basic 'diffuse' lesion usually showed a generalised capillary wall thickening, which was most marked in the axial regions, although one patient showed no significant abnormality. Capillaries were often dilated. Diabetic nodules, which formed on progression of the disease process, were rounded or ovoid hyaline, eosinophilic, laminated structures, situated at the periphery of the glomerulus. On electron microscopy the diffuse lesion showed a uniform increase in the thickness of the basement membrane, leading to axial basement membrane accumulation. The nodules appeared as an agglomeration of small masses of basement membrane.

The glomerular appearances were classified according to the severity

Fig. 43. Correlation of selectivity values and glomerular changes.

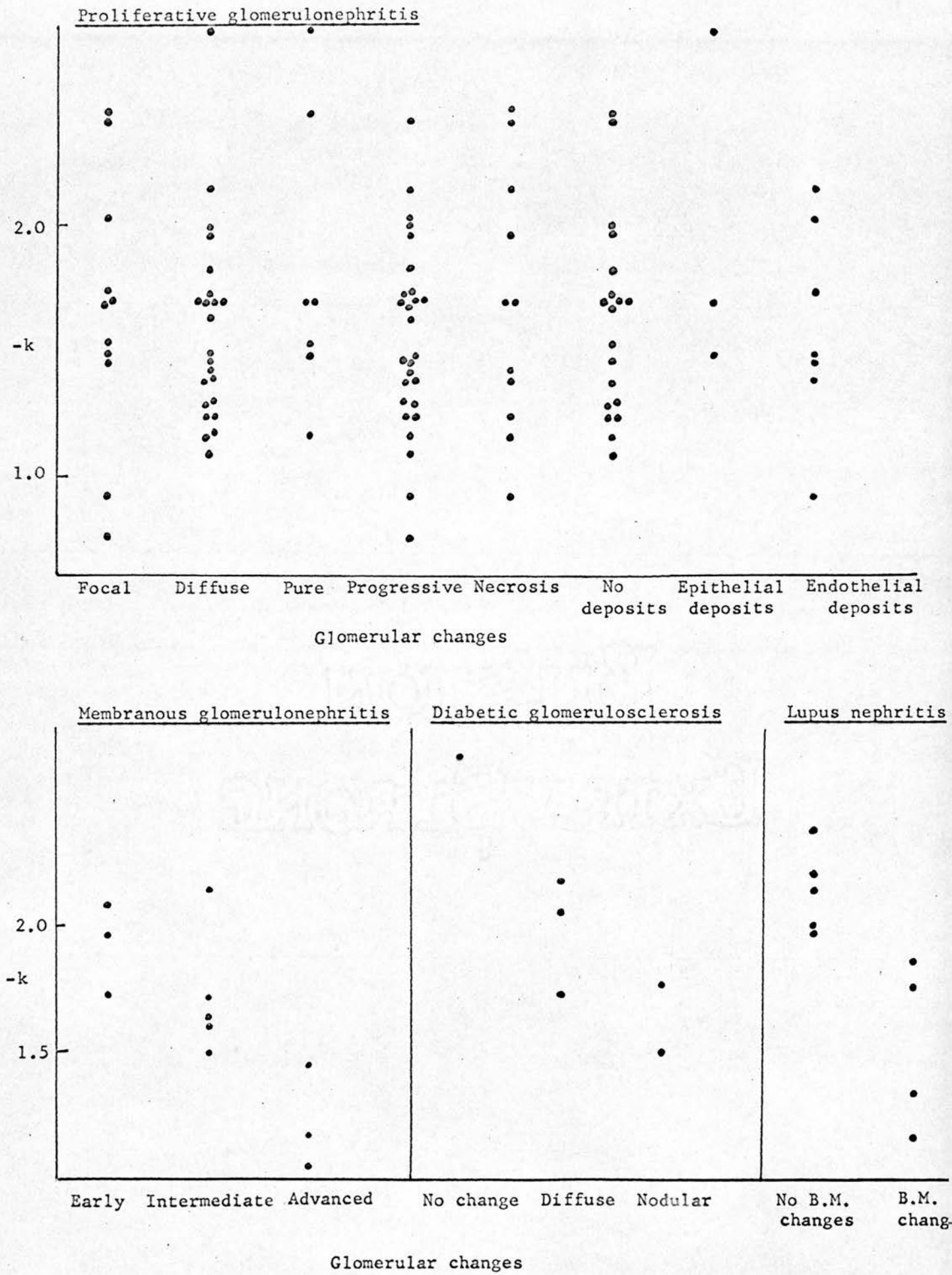
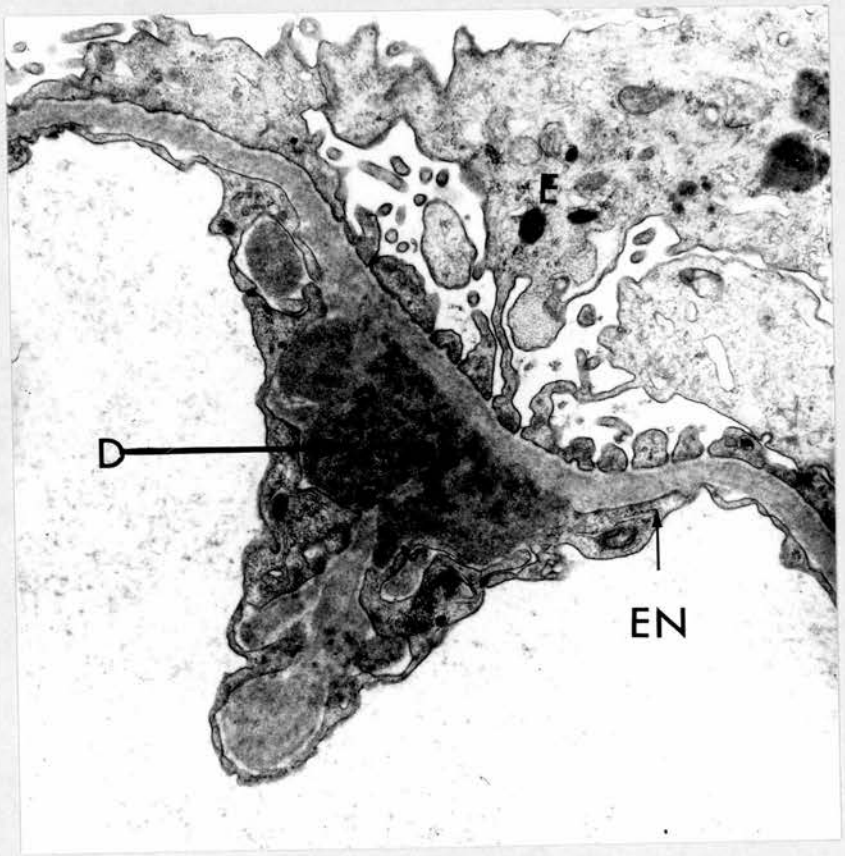


Fig. 44. Lupus nephritis.

Pedicels, in this case, are well preserved. The basement membrane appears normal. There is a large osmiophilic deposit in a sub-endothelial position.

Magnification x 15,000.



of the disease process into those showing no significant changes, moderate diffuse changes and advanced nodular changes. When plotted against selectivity values there was a correlation (Fig. 43), with lower selectivity values associated with an increasing degree of glomerular damage.

Lupus nephritis

Selectivity values ranged from 1.17 - 1.84.

A considerable variety of changes could be seen on light microscopy in this condition. Commonly the appearances were of a focal glomerulonephritis, occasionally associated with peripheral patches of glomerular necrosis and foci of fibrinoid change in capillary walls. On electron microscopy (Fig. 44) the main features were those of a proliferative glomerulonephritis, with variable smearing of epithelial cell foot processes.

The presence of osmiophilic deposits between the basement membrane and endothelial cells is often regarded as characteristic of this syndrome. This feature has, however, also been observed in cases where a diagnosis of DLE was not supported by other findings, and in the present series with lupus nephritis, only 2 of the 9 patients examined by electron microscopy showed such deposits. Selectivity values in these cases were 1.34 and 1.77. In two other patients with selectivity values of 1.17 and 1.87 the basement membrane showed thickening or a dark material, which may have been an early form of deposit. The mean selectivity value for patients with basement membrane changes was therefore 1.54, and for the remainder was 2.16, the ranges of values being 1.17 - 1.87 and 1.99 - 2.40 respectively (Fig. 43).

Membranous glomerulonephritis

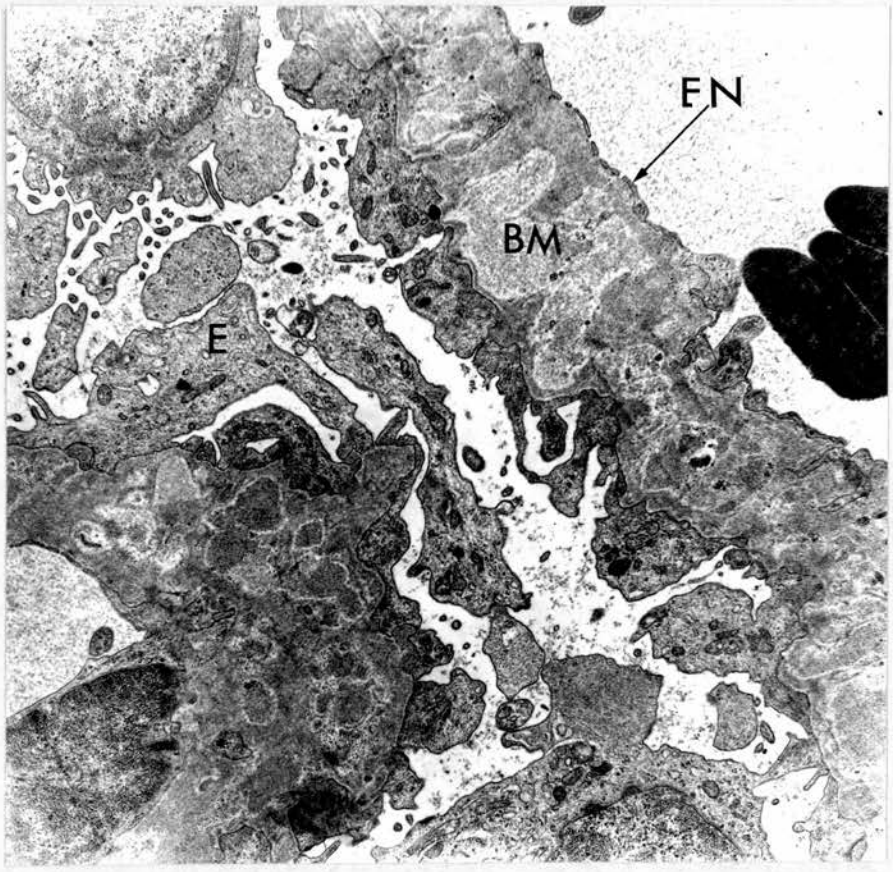
Selectivity values ranged from 1.04 - 2.15, mean 1.64.

Glomeruli on light microscopy showed significant changes. Capillary walls were diffusely thickened. Endothelial cells appeared enlarged and

Fig. 45. Membranous glomerulonephritis.

There is marked loss of pedicel structure. The endothelium appears normal. The basement membrane area is grossly thickened and contains both light and dark vacuolated areas.

Magnification x 7,000.



there was only minor endothelial swelling and proliferation. On electron microscopy the changes seen in glomeruli varied according to the stage of the disease. In almost every case there was complete smearing of the epithelial cell foot processes, and only minor endothelial cell swelling. The basement membrane showed the most characteristic changes, with accumulations of hyperosmiophilic material between the basement membrane and epithelial cells, irregularity and corrugation on the epithelial side, and the development of vacuoles within the abnormal osmiophilic material and the membrane proper. As the condition progressed focal protusion of epithelial, and to a lesser extent endothelial, cell cytoplasm into the abnormal basement membrane was seen. In the later stages islands of cytoplasm occurred within the basement membrane. Advanced changes were associated with capillary narrowing, causing complete obliteration of the capillary lumen. Fig. 45 shows part of a glomerulus at an intermediate stage of the disease.

The glomerular appearances were classified according to the stage of the disease process, into those showing "early","intermediate" and "late" changes. When plotted against selectivity values there was a significant correlation (Fig. 43), with lower selectivity values associated with an increasing degree of glomerular damage.

Proliferative glomerulonephritis

Selectivity values ranged from 0.76 - 2.78, mean 1.62.

The most striking feature of the condition, before progression occurred, was the increase in glomerular cells, almost always of the endothelial type. This proliferation was focal or diffuse and in some cases glomeruli appeared to be enlarged and hyperlobulated. Further changes such as crescent formation and adhesion to Bowman's capsule were sometimes

Fig. 46. Proliferative glomerulonephritis.

There is an increased number of endothelial cells within the capillary loop, which also contains a white blood cell. Epithelial cell pedicels show moderate smearing. The basement membrane is relatively normal, but shows some proliferation round the middle endothelial cell.

Magnification x 5,300.

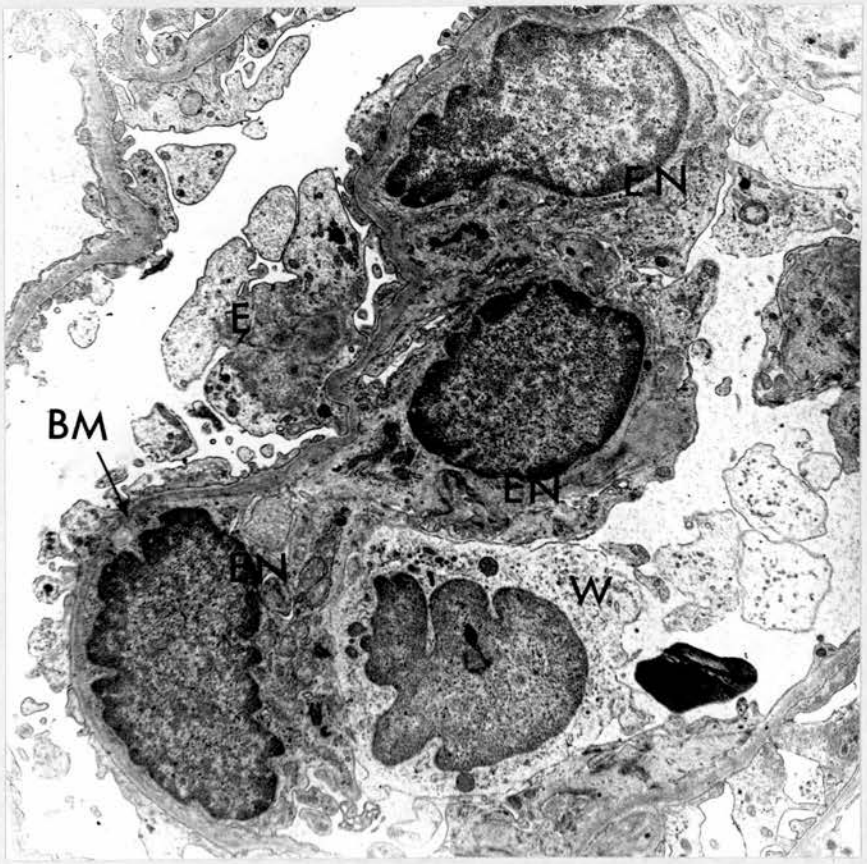
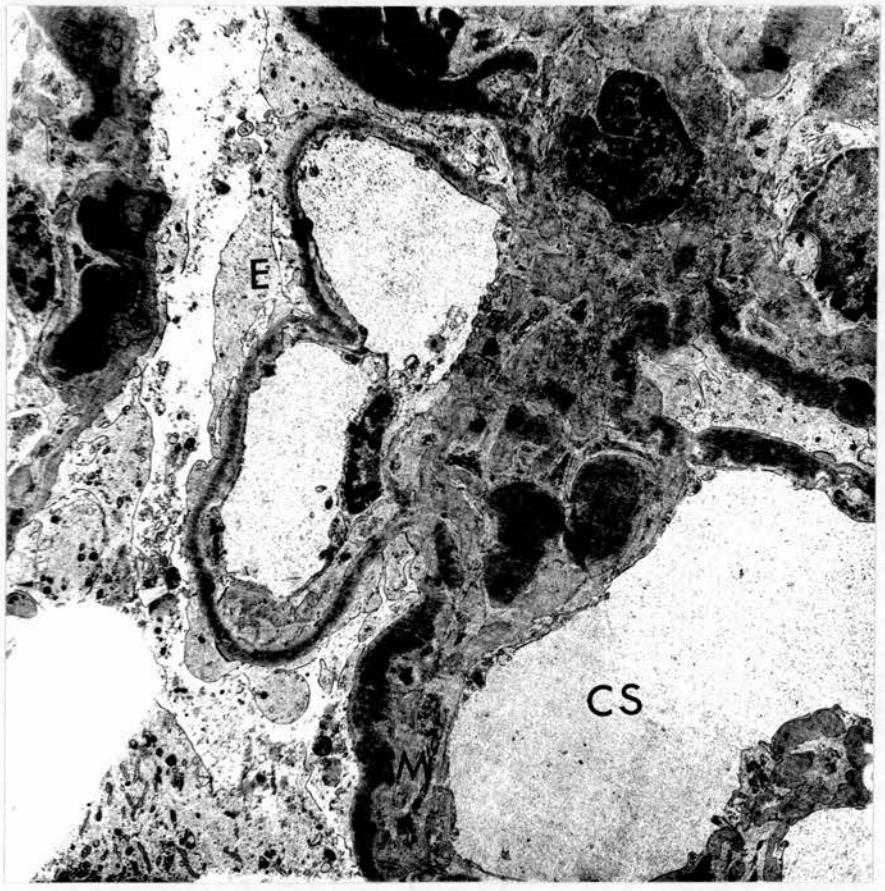


Fig. 47. Mixed membranous and proliferative glomerulonephritis

Normal pedicel structure is lost. The basement membrane is unevenly thickened by the deposition of osmiophilic material within its substance.

Magnification approx. x 1,500.



superimposed. When the lesion was progressive this was commonly expressed by a fibrillar increase in basement membrane material, leading to capillary wall thickening. These appearances were confirmed on electron microscopy (Fig. 46). In progressive cases branching and duplication of apparently normal basement membrane material was seen. In addition a few cases showed the presence of granular deposits on either the epithelial or endothelial aspects of the basement membrane. The significance of these is not clearly established, but they are regarded by some workers as being involved in an immunological process.

Glomerular abnormalities were classified according to whether they showed pure or progressive and diffuse or focal changes. The results are plotted against selectivity in Fig. 43, which also shows the selectivity values of patients in whom areas of focal necrosis were seen, and in whom epithelial and endothelial basement membrane deposits were seen. There was no obvious correlation of selectivity with any of the glomerular changes.

Mixed membranous and proliferative glomerulonephritis

Selectivity values ranged from 1.40 - 1.70, mean 1.56.

Glomeruli on light microscopy showed enlargement and hyperlobulation, with endothelial cell proliferation; the distribution of proliferation varied from diffuse to focal in individual glomeruli. Marked, but focal, capillary wall thickening was seen, sometimes progressing to hyaline obliteration of the structure in that region. On electron microscopy (Fig. 47) the most striking feature was the dark basement membrane material, which was suggestive of early osmiophilic deposits.

The number of patients in this group was too small to relate individual selectivity values to ultrastructural appearances.

Fig. 48. Acute ischaemic renal failure

Fibrillar material is seen within the capillary loop. Although it is not possible to demonstrate the periodicity of fibrin, it is assumed that this material indicates incipient thrombosis.

Magnification x 29,000

Chronic glomerulonephritis

Selectivity values ranged from 1.00 - 1.69, mean 1.28.

The appearances could, in some cases, be related to the type of glomerulonephritis originally present, but in all cases the glomerular changes were sufficient to obliterate most of the organized structure. Partial or complete hyalinisation was sometimes seen, or alternatively destruction was caused by progressive capillary wall thickening. Renal biopsy tissue was not examined by electron microscopy in these cases.

Glomerular changes did not show sufficient differences for a comparison of selectivity and ultrastructural changes to be carried out.

Postural proteinuria

Selectivity values ranged from 0.75 - 1.38, mean 1.11.

Light microscopy revealed no significant abnormality. On electron microscopy some abnormal features were found in all cases. The epithelial cells showed focal areas of foot process fusion, ranging from simple broadening of pedicels to the presence of a continuous epithelial layer applied to the basement membrane. Patchy, irregular, "ballooning" of epithelial cell cytoplasm was also seen. Small dark bodies resembling Folli bodies were noted in the glomerular space, in close association with the epithelial cells. The basement membrane was normal in appearance and thickness and the endothelial cells showed no significant changes.

Glomerular changes were very similar in all the patients with postural proteinuria and therefore no comparison of selectivity and ultrastructural changes was carried out.

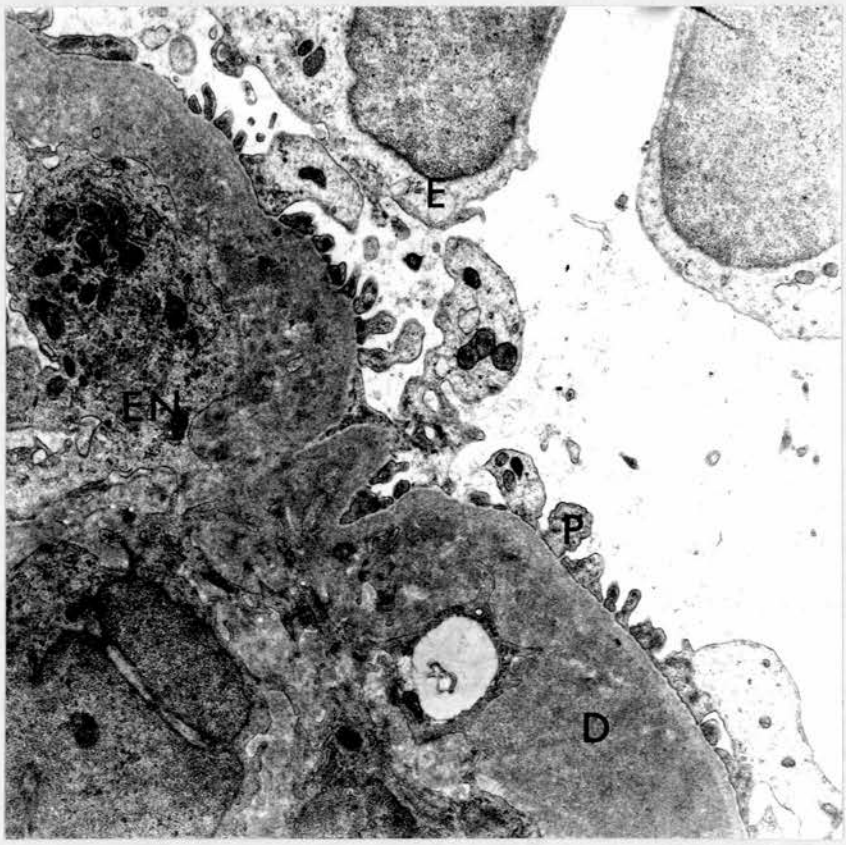
Acute ischaemic renal failure

Selectivity values ranged from 0.66 - 1.20, mean 0.90.

Light microscopy revealed no significant glomerular abnormality,

Fig. 49. Toxaemia of pregnancy

Pedicel structure is well preserved. Endothelial cells are swollen, giving rise to narrowing of the capillary lumen. A dense deposit is seen in a sub-endothelial position.



although tubular necrosis was present in all cases. On electron microscopy some glomeruli were normal in appearance, but in others a heavy granular deposit within the capillary was seen (Fig. 48). This material was seen in 2 patients to have an organized structure in places, resembling fibrin. Excessive aggregates of platelets were also occasionally seen. The endothelium, basement membrane and epithelium were consistent with normal appearances.

Glomerular changes were very similar in all the patients with acute ischaemic renal failure and therefore no comparison of selectivity and ultrastructural changes was carried out.

Miscellaneous

The three patients with toxaemia had selectivity values ranging from 1.13 - 1.50, mean 1.33. On light microscopy endothelial cell enlargement was striking, and in parts the basement membrane showed a patchy fibrillar appearance. On electron microscopy (Fig. 49), the endothelium appeared swollen, with areas suggestive of sub-endothelial osmiophilic deposits. The pedicel structure and basement membrane were normal.

The two patients with renal vein thrombosis had selectivity values of 1.95 and 1.75 and light microscopy showed changes of focal glomerulitis. On electron microscopy mild proliferative changes were seen in the more selective patient, and the other showed changes characteristic of early membranous glomerulonephritis.

Two of the three patients with potassium depletion and hypertension had a renal biopsy. Selectivity values in this group ranged from 2.16 - 2.20, mean 2.18. On light microscopy slight proliferation of endothelial cells was seen. Statis in capillary loops was the only abnormality seen on electron microscopy.

The patient with myelomatosis had a selectivity value of 1.44. The glomeruli appeared fairly normal, with some enlargement of cells. The patient with pyelonephritis had a selectivity value of 1.43 and the glomeruli showed varying degrees of hyaline replacement of their structure. Entirely normal glomeruli were found in the patient with exercise haematuria, selectivity value 0.90.

The two patients with post-partum renal disease had selectivity values of 1.02 and 1.83. The glomerular changes were striking and on light microscopy proliferation of cells and thickening of capillary walls was noted. On electron microscopy there was complete epithelial smearing and a prominent and unusual lesion between the basement membrane and endothelial cells.

Four patients had proteinuria of unknown origin. In two patients a thickened basement membrane was seen on electron microscopy; the selectivity values were 1.54 and 1.80. In one patient with a selectivity value of 1.70 some hypertensive changes were seen, but the appearances were otherwise normal. In the fourth patient some statis in capillary loops and folding of the basement was seen, but the appearances were otherwise normal; the selectivity value was 2.10.

Summary

High selectivity values were associated with minimal glomerular damage in minimal lesion glomerulonephritis, but with marked glomerular changes in amyloid disease. In diabetic glomerulosclerosis, lupus nephritis and membranous glomerulonephritis mean selectivity values were intermediate and glomerular changes were significant. The degree of damage correlated with the selectivity in these diseases, a low selectivity value being associated with an increasing degree of glomerular damage. In proliferative glomerulo-

nephritis there was a very wide range of selectivity values, and obvious variations in the degree and type of lesion, but no correlation between the two was found. In mixed membranous and proliferative glomerulonephritis the mean selectivity value was lower than in proliferative or membranous glomerulonephritis and significant basement membrane changes were seen. Chronic renal failure showed obliteration of glomerular structure and low selectivity values. Very low selectivity values, but minimal glomerular damage, similar to that seen in minimal lesion glomerulonephritis, were found in postural proteinuria. Almost normal glomeruli were seen in acute ischaemic renal failure, and selectivity values in this disease were the lowest of the whole group of patients studied.

TABLE 37.

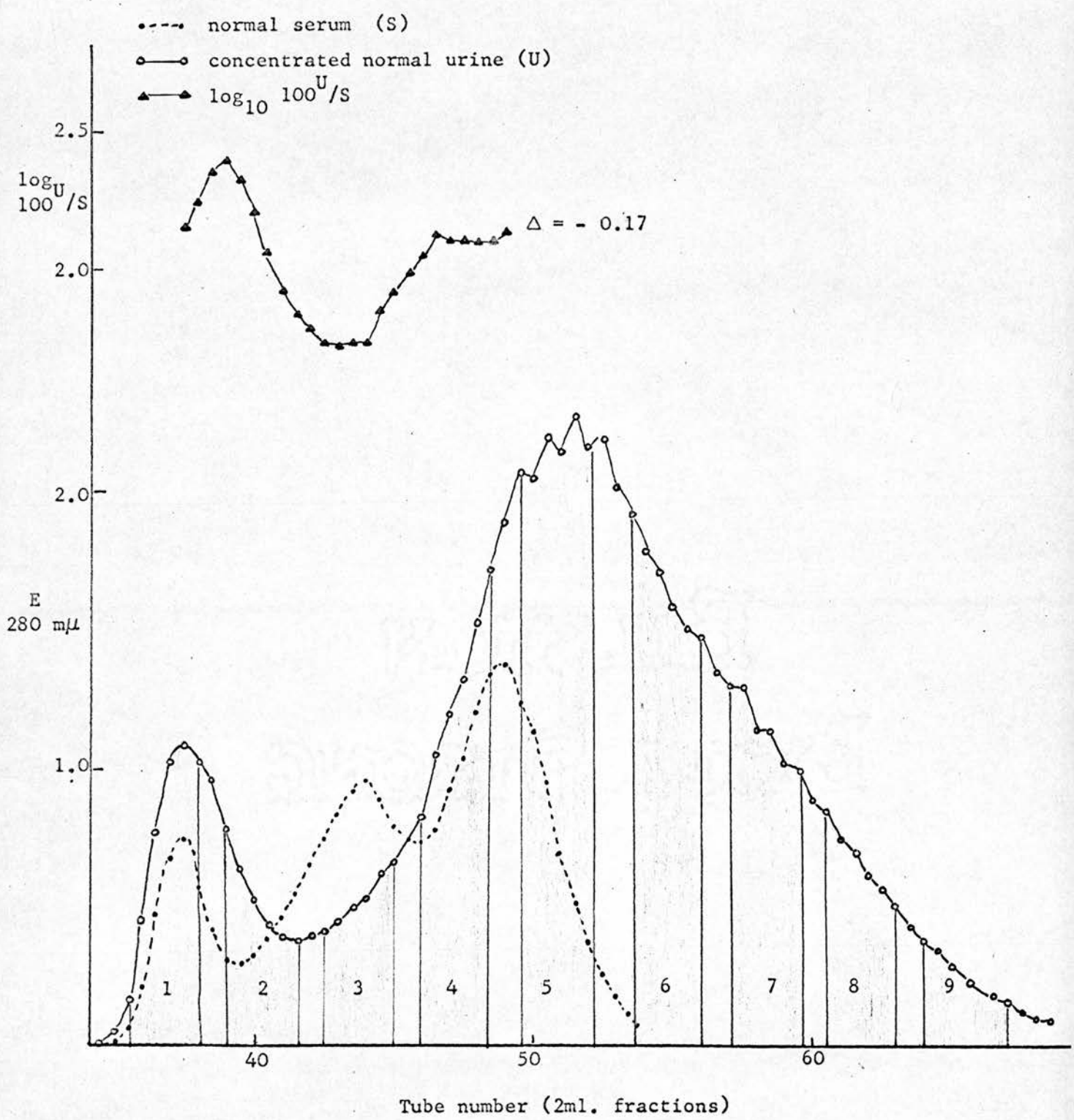
Protein indices of selectivity by immunodiffusion in normal subjects

Indices of selectivity (-k), albumin serum:urine ratios (Alb S:U) and relative clearances of individual proteins (transferrin (S), γ-globulin (γ), α_2-macroglobulin (α_2) and β -lipoprotein (β)) in 10 normal subjects.

Subject	Alb S:U	S	γ	α_2	β	-k
1	5120	143	125	14	<7	0.09
2	10750	50	120	<4	-	>1.28
3	2560	100	84	27	13	0.59
4	8750	100	117	6.3	<3	1.18
5	8830	67	166	2.3	<3	1.56
6	8250	59	150	9.0	<2	0.96
7	3200	72	42	4.2	<1	1.28
8	10900	112	140	2.9	-	1.54
9	6160	100	100	16	<2	0.78
10	1600	112	69	5.0	<1	1.29
Mean	6610	92	111	8.7		1.14

Fig. 50. Determination of selectivity of normal proteinuria by gel filtration.

The shaded areas represent fractions which were examined by immunoelectrophoresis. (see Fig. 51)



3.3. VALUES OF SELECTIVITY IN NORMAL SUBJECTS

Immunodiffusion

Selectivity values were estimated on 10 normal healthy subjects by immunodiffusion. The results are shown in Table 37, together with the relative clearances of the individual proteins and the serum:urine albumin ratios. The clearances of γ-globulin were much higher than in cases of proteinuria and in 7 of the 10 subjects were higher than the albumin clearance. β-Lipoprotein was only detected in one urine. Indices of selectivity ranged from 0.78 - 1.56, mean 1.14 and albumin serum:urine ratios ranged from 1600 - 10900, mean 6610.

Gel filtration

Selectivity values were estimated on 2 normal healthy subjects by gel filtration. In addition a third estimation was made using a normal urine pool and a normal serum pool.

Selectivity values for the two individuals were + 0.49 and - 0.11. Albumin serum:urine ratios were 7200 and 2100 respectively. The correlation coefficient for the log-log plots of renal clearance against molecular weight were r =+0.919 and r =-0.256 respectively . The urine pool had a selectivity value of - 0.17, correlation coefficient r =-0.242. The urines all had a similar elution pattern on G 200 and Fig. 50 shows the elution pattern of the serum and urine pools and the plot of $\log_{10} 100 \frac{\text{urine}}{\text{serum}}$ against tube number. The urine had large first and third peaks and a considerable proportion of protein eluting after the third peak.

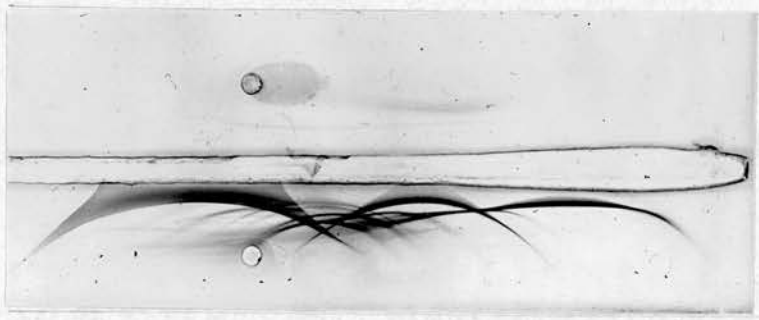
Immunoelectrophoretic examination of gel filtration elution patterns

The elution patterns of the 3 urines, on which selectivity values were obtained by gel filtration, were examined by immunoelectrophoresis.

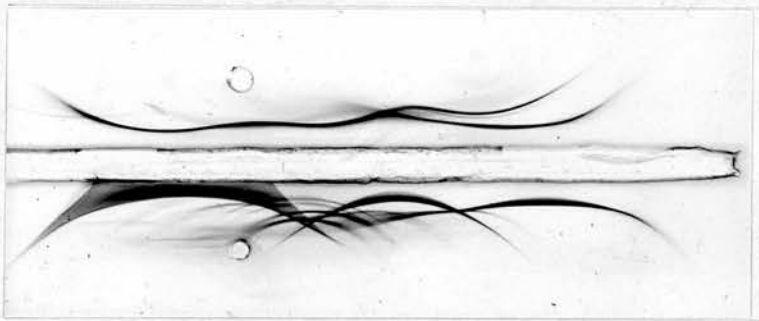
Fig. 51. Immunoelectrophoretic examination of normal urine proteins after gel filtration.

The fractions examined correspond to the fractions shown in Fig. 50. Each urine fraction (U) was compared immunoelectrophoretically to normal serum (S).

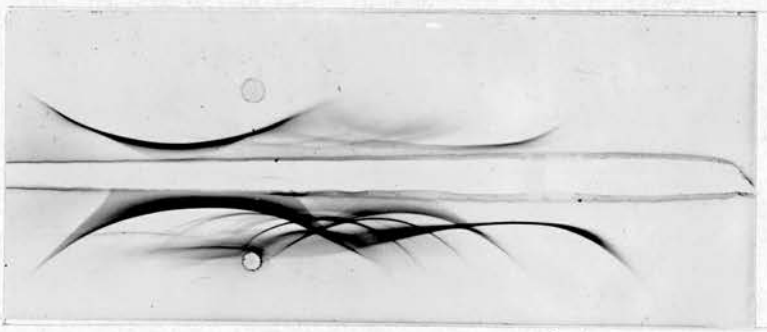
Fraction U1



Fraction U3



Fraction U5



Fraction U7

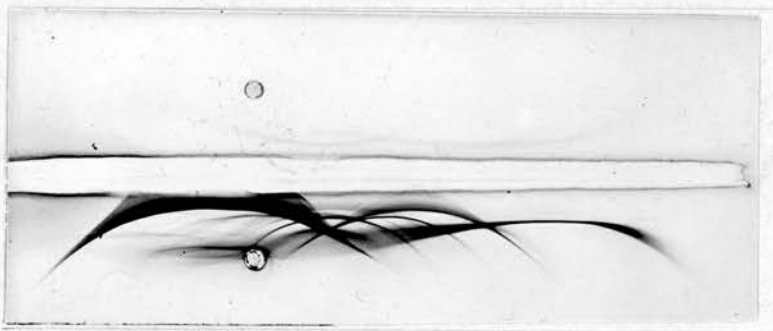


TABLE 38.

Dextran indices of selectivity in normal subjects.

Dextran indices of selectivity (D), creatinine clearance (Cr Cl) and total urine protein (UP) in 6 normal subjects.

* Subject 2 had one kidney.

Subject	D	Cr Cl ml./min	UP mg./24 hr.
1	2.98	-	21
2	2.76	37*	87
3	2.50	-	-
4	2.38	93	14
5	3.65	92	15
6	3.19	100	45
Mean	2.91		

The results were similar. Fig. 50 shows the fractions that were examined from the normal urine pool and Fig. 51 shows the immunoelectrophoretic separations of some of them.

Few of the serum components were identified in the urine, despite the fact that large amounts of protein were eluting over the serum protein range. In one case γ_{1M}-globulin and α_2-macroglobulin were found in the first urine peak, and in all the urines a long $\alpha_2\beta$-line and an additional β-line were also found in the first peak. At the second serum peak γ-globulin and a faint albumin peak were visible in the urine, together with a long α-β-line. At the third serum peak γ-globulin and a strong albumin line were seen and in addition some unidentified α_2- and β-lines were visible. The urine proteins continued to be eluted towards lower molecular weights than the serum protein, but the only components that were found were an α_2-line and γ-globulin.

Dextran indices of selectivity

Dextran indices of selectivity were estimated on 6 subjects with normal renal function, although most of them were hospital patients. The results are shown in Table 38, together with their creatinine clearances and total urine proteins. The selectivity values were all very high and ranged from 2.38 - 3.65, mean 2.91.

Correlation of selectivity values

Selectivity values by immunodiffusion were low, with a mean of 1.14. Using gel filtration the proteinuria was found to be very much more unselective, and even negative values were obtained in 2 out of 3 samples. There was therefore a lack of correlation between the methods, since immunodiffusion indicated an unselective proteinuria, but gel filtration suggested that there was no selectivity at all. The factor of 0.67, which

related immunodiffusion and gel filtration values in proteinuria would give a mean gel filtration in normal proteinuria, calculated from the immunodiffusion value, of 0.77.

Immunoelectrophoretic examination of the urine elution patterns on G 200 in all 3 cases indicated that not many identifiable proteins derived from the serum were being estimated in the urine. There were, however, several unidentified components, which reacted with anti-human serum to form precipitin arcs in α_2- and β-regions.

The values of protein selectivity obtained by immunodiffusion were uniformly low, whereas those of dextran selectivity were uniformly high. There was therefore no correlation between the different methods of estimating selectivity.

4. DISCUSSION

4.1 Discussion of results

4.1.1 Protein selectivity

4.1.2 Renal permeability to other macromolecules

4.1.3 Selectivity, diagnosis and glomerular ultrastructure

4.2 General discussion

4.2.1 Implications of selectivity studies

4.2.2 Value of selectivity estimations

The results are discussed in two sections. In the first the results of the selectivity studies and the relationship of selectivity and glomerular ultrastructure are discussed, and the second contains a general discussion of the value and implications of estimations of selectivity, particularly in renal disease.

4.1. DISCUSSION OF RESULTS

4.1.1. PROTEIN SELECTIVITY

Values of protein selectivity in health and disease

Estimation of relative clearances of individual proteins of different molecular size was first reported by Blainey et al. (1960), using an immunodiffusion method. The same technique was used in this study, and the linear relationship between relative clearance of protein and molecular weight on a log-log scale has been confirmed. Results have been expressed as indices of selectivity (-k), or the slope of the line relating relative clearance of protein to molecular weight on a log-log plot. The accuracy and limitations of the method have been investigated, and although the method is expensive, because of the cost of antisera, it was found to be reproducible and suitable for a large series of estimations.

Immunoelectrophoresis has also been shown to be a useful qualitative method of assessing the selectivity of proteinuria. The good correlation with the immunodiffusion method demonstrated that different selectivity patterns can readily be distinguished by immunoelectrophoresis, provided both the number and molecular weight of the proteins identified were considered. The alternative method which was developed, for estimating indices of selectivity (Δ) by gel filtration on Sephadex G 200, also proved to be of value. The method is simpler than the immunodiffusion one and is less expensive in terms of materials. It is, however, more time-consuming and slightly less accurate.

Gel filtration and immunodiffusion are methods which differ considerably in principle. Both have inherent limitations. Selectivity by immunodiffusion is estimated from the relative clearances of five individual

serum proteins. Although only proteins derived from the serum are measured, the method is unable to distinguish protein fragments with antigenic activity from the parent protein molecules. Gel filtration, on the other hand, measures relative clearances of mixtures of proteins of known gradation in molecular size, but no distinction can be drawn between protein of serum origin and protein from the urinary tract.

An attempt was made by Hardwicke (1965), to correlate the relative clearances of individual proteins obtained by immunodiffusion with their relative clearances by gel filtration. Hardwicke did not, however, indicate how individual protein clearances were determined using Sephadex G 200; it would seem for the purposes of the calculations that he assumed that each individual protein was eluted at a single tube. The basis for his choice of tube number is not clear from his paper. Since any protein elutes from Sephadex over a number of tubes, and since a single tube contains a mixture of proteins, the validity of gel filtration as a method for determining the clearance of an individual protein is dubious.

In this study, although the units of Δ and -k are not the same, they are both based on the linear relationship between relative renal clearance of protein and molecular weight on a log-log plot. The index of selectivity by immunodiffusion, -k, is the change in renal clearance of protein per unit change in molecular weight, whereas the index of selectivity by gel filtration, Δ, is the total change in renal clearance of protein over a fixed range of molecular weight. It is therefore valid to compare Δ and -k.

The excellent correlation between results obtained by the two methods in proteinuria indicates that the limitations of the techniques are only theoretical, and that both immunodiffusion and gel filtration are measuring proteins of serum origin, which are not degraded to smaller molecular weight

fragments. This was experimentally confirmed by immunoelectrophoretic examination of serum and urine elution patterns obtained on gel filtration. Selectivity of proteinuria therefore can be assumed to be a meaningful estimate of renal permeability.

In renal disease with proteinuria, estimates of renal permeability in a population of 130 patients had a normal type of distribution, with values ranging from an almost total lack of selectivity to a high degree of selectivity ($-k = 0.40 - 3.82$, $\Delta = 0.40 - 2.51$). Recently Joachim et al. (1964), using immunodiffusion, have investigated 48 patients with several different types of renal disease with similar results ($-k = 1.00 - 3.08$). Hardwicke (1965) has described the use of gel filtration to assess renal permeability to protein in 10 patients. His results are not directly comparable, as he used the slope of the line relating $\log_{10}$ 100 urine/serum to tube number as his index of selectivity. However, multiplication of the slopes by the average number of tubes used in this calculation gives similar values for Δ ranging from 0.7 to 2.4.

When the degree of proteinuria is slight, selectivity must be interpreted more cautiously in terms of renal permeability, since the limitations discussed above become of practical significance, particularly when gel filtration is employed. In proteinuria of under 1 g./day, indices of selectivity obtained by gel filtration showed a poor correlation with values obtained by immunodiffusion. The anomalously low gel filtration values are most likely to be due to the presence of a relatively large contribution of urinary tract protein (Grant, 1957, 1959) to the total urinary protein; other possible sources are discussed later (4.2.1). Gel filtration is therefore of limited value in the estimation of selectivity where the urine protein is under 1 g./day.

Indices of selectivity obtained by immunodiffusion sometimes showed changes when the proteinuria was very slight. However, in the patients with postural proteinuria and acute ischaemic renal failure, in whom the selectivity was low, there was no significant change in value when the proteinuria fell to trace and normal levels in the course of clinical recovery. Any change in the mechanism or origin of the proteinuria was presumably masked by the similarity of the selectivity patterns in these diseases and in health. Changes in selectivity values were seen only in selective patients, in whom the selectivity fell towards the unselective values found in normal subjects. Significant reductions in selectivity values occurred approximately at albumin serum:urine ratios of over 400. The ratio of 400 approximates to a total urine protein of 200 mg., assuming the serum albumin concentration to be 4.0 g.%, the urine volume to be one litre and the urine protein to contain 50% albumin. The criteria of proteinuria for this study, which was taken as an albumin serum:urine ratio of over 400 or a total urine protein of over 200 mg., was therefore a reasonable one. The immunodiffusion method, as an estimate of renal permeability, is certainly valid up to albumin serum:urine ratios of 200, and appreciable reductions in selectivity were not introduced up to ratios of 400.

In normal subjects, the low indices of selectivity obtained by immunodiffusion indicate the normal kidney could be unselective in the excretion of protein molecules. However, since selectivity values are significantly reduced in selective patients who completely recover, the mechanism of normal proteinuria must be different from that of proteinuria in renal disease. The origin of normal proteinuria is discussed more fully later (4.2.1). Rowe and Soothill (1961a) also showed that normal proteinuria was unselective,

although they found lower clearances of α_2-macroglobulin and no urinary β-lipoprotein. The clearances of ϒ-globulin, in both this series and that of Rowe and Soothill, were higher in normal subjects than in patients with proteinuria of a comparable selectivity. This was probably due to the low molecular weight ϒ-globulins, known to be present in normal urine (Franklin and Kunkel, 1957; Cornillot et al., 1963; Fahey, 1963), cross reacting with the anti-7S ϒ-globulin. Low molecular weight ϒ-globulin reacting with anti-7S ϒ-globulin and eluting after the albumin on gel filtration was detected in normal urine in this study, supporting this view.

The lack of correlation of selectivity values obtained by gel filtration and immunodiffusion in normal subjects suggested that, as with proteinuria of under 1 g./day, urinary tract protein makes a significant contribution to the total urine protein. This was confirmed by immuno-electrophoretic examination of the normal urine elution pattern from Sephadex, when only a few identifiable serum components and a few unidentified α_2- and β-globulins, which reacted with anti-human serum, were found. Normal selectivity values obtained by gel filtration could not therefore be interpreted in terms of renal permeability.

Studies on protein selectivity in renal disease

Squire, Hardwicke and Soothill (1962) have commented that when proteinuria persists selectivity values are remarkably constant. Although variations in selectivity values in renal disease were found over periods of 1 year, these were almost all within the estimated physiological variation, and this stability of the selectivity persisted, even when renal function and, in particular, urine protein levels were rapidly changing, either spontaneously or as a result of steroid treatment. Vere and Walduck (1966), using electrophoretic and gel filtration techniques, and Joachim et al. (1964)

have also recently shown that there is no significant change in renal permeability to protein during steroid induced remission of the nephrotic syndrome. In addition, the commencement of steroid treatment was found in this study to have no significant effect on selectivity values, with the exception of three responsive selective patients, in whom values significantly increased.

Marked clinical and histological deterioration in patients with chronic renal disease usually occurs over periods of years. In this study the data was not sufficient to draw any conclusions as to long term stability of selectivity values. Patients studied for up to 2 years rarely had decreases in selectivity values of over 20%. It may, however, be of some significance that more patients had a mean decrease than a mean increase in selectivity/six months, and also that a higher proportion of patients with a mean increase were being treated with steroids.

Responsiveness to steroids and ACTH has been investigated by Lauson et al. (1954) and McCrory, Rapaport and Fleisher (1959), by measuring albumin clearances in relation to G.F.R., with promising results. In this study, however, the relationship between steroid response and selectivity was very much more striking. This association of a highly selective proteinuria with response to steroid treatment was first suggested by Blainey et al. (1960), and has recently also been confirmed by Joachim et al. (1964) and Vere and Walduck (1966). It has been suggested that patients with minimal lesion glomerulonephritis respond best to steroids (Vernier et al., 1958). Although in this study patients with this disease had the most selective type of proteinuria, there were other types of renal disease with overlapping ranges of selectivity values. It was of great interest that some of these highly selective patients with proliferative glomerulonephritis and lupus

nephritis were also found to respond to steroids.

Although spontaneous abolition of proteinuria in the present control group of patients was infrequent, it was significant that the two patients who partially and fully responded also had selective proteinuria and both suffered from ~~mixed membranous and proliferative glomerulonephritis and~~ proliferative glomerulonephritis.

Children with the nephrotic syndrome often have a minimal lesion glomerulonephritis and a selective proteinuria (Cameron and White, 1965), and Cameron (1966) found the selectivity fell with increasing age. Few children were included in this study, however, and amongst the adult population with renal disease there was a significant positive correlation between selectivity and age. The association of high selectivity values with increasing age may be a result of the association of high selectivity values with less severe glomerular damage (3.2.5), and hence better prognosis. There was no correlation between sex of the patient and selectivity values.

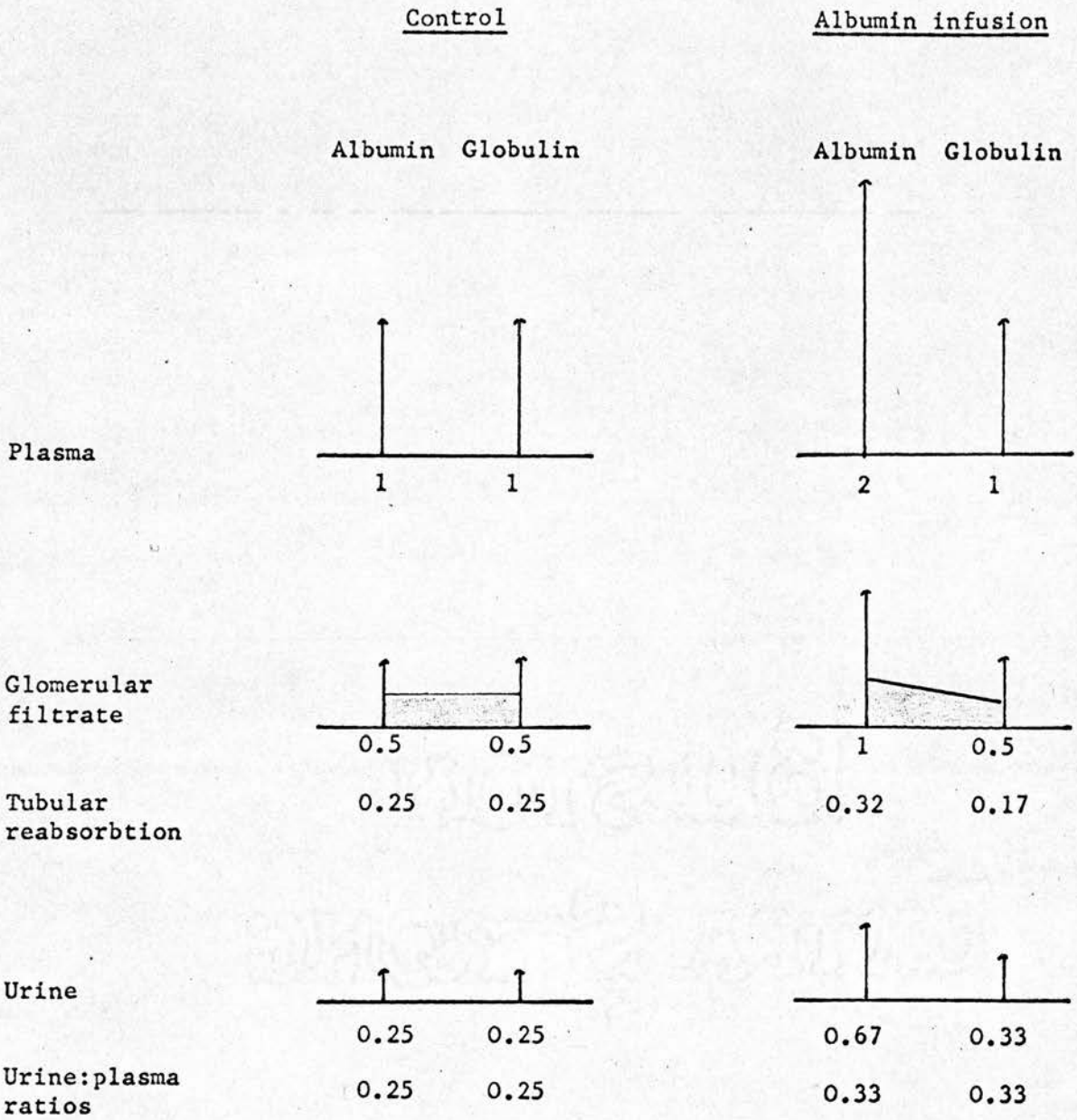
Joachim et al. (1964) found no correlation between total protein excretion and selectivity, but a significant correlation between renal function and selectivity. In this study selectivity was also found to have no correlation with total protein excretion. There was, however, only a degree of correlation between selectivity and renal function; this was largely accounted for by a complete lack of correlation in patients with proliferative glomerulonephritis, and amyloid disease.

Selectivity on infusion of albumin

In theory, if there is a competitive process for tubular reabsorption of protein, similar to the reabsorption of amino acids, then the relative concentrations of protein in the urine are the same as their relative concentrations in glomerular filtrate. The reabsorption is in proportion

Fig. 52. Diagrammatic representation of competitive tubular reabsorbtion of protein.

Arbitrary units are shown. Tubular reabsorbtion is assumed to be competitive and working at a maximum of 0.5 units. It is indicated by the shaded areas.



to the amount of each protein present. If the plasma concentration of albumin is increased, a correspondingly increased amount of albumin will be filtered by the glomerulus. Although the plasma:glomerular filtrate ratio remains the same, the actual amount of albumin in the glomerular filtrate is increased. In proteinuria, tubular reabsorption is at a maximum and if it remains competitive the amount of albumin reabsorbed will be increased, and the amount of globulin reabsorbed will therefore be decreased. The result is that the amounts of both albumin and the globulins in the urine are increased. The relative urine:plasma ratios, however, remain the same. The situation is shown diagrammatically in Fig. 52. In these circumstances the selectivity pattern should also remain the same.

If individual proteins have different reabsorption systems, then increasing the albumin in the glomerular filtrate would simply increase the albumin in the urine, with no effect on the globulin excretion. The selectivity value in this case would rise.

The present study was carried out on a small number of patients. However, in the two patients with major proteinuria no rise in selectivity was observed and there was no change in selectivity in one unselective patient. The reduction in selectivity in the selective patient is more difficult to interpret, but it could not have been caused by a non-competitive tubular reabsorption mechanism. It could theoretically have resulted from an increase in glomerular permeability, following the albumin infusion. Malmendier, Koster and Lambert (1960) have demonstrated increased albumin clearances on expansion of plasma volume, and they concluded that this was due to an increase in the size of the glomerular pores. Alternatively, saturation of a new nephron population could produce this effect, but the new glomeruli would require to have a less selective permeability.

The rise in selectivity in the patient with acute ischaemic renal failure may have been due to a different tubular reabsorptive mechanism, as the main histological lesion in this disease is tubular necrosis. Alternatively, the large amount of albumin infused in this experiment could have saturated the tubular reabsorption in some normal nephrons. If the normal glomerulus is highly selective, as indicated by the dextran studies, then this would result in a rise of selectivity. The origin of the proteinuria in acute ischaemic renal failure is discussed later (4.1.3).

4.1.2. RENAL PERMEABILITY TO OTHER MACROMOLECULES

Other individual proteins

The correlation of the relative clearances of ceruloplasmin, γ_{1A}-globulin and α_1-lipoprotein with selectivity values gives additional confirmation of the linear log-log relationship of renal clearance of protein and molecular weight. Although γ_{1A}-globulin is immunologically related to 7S- γglobulin (Heremans, Heremans and Schultze, 1959), the degree of cross reaction may not be appreciable, and there is no reason to doubt the validity of the γ_{1A}-globulin clearances. Clearances of orosomucoid probably also correlate with selectivity; this has been confirmed by Joachim et al. (1964).

Two out of eleven proteins were found to deviate from the linear relationship, namely prealbumin and fibrinogen, although clearances of both proteins were of the expected order of magnitude. Prealbumin has the most rapid electrophoretic mobility of all the plasma proteins; this is related to its high tryptophan content. In addition, it has a carbohydrate content which is very different from the average of all plasma proteins (Winzler, 1960). Little is known about the mechanism of glomerular transport of plasma proteins, but it is possible that the unusual chemical composition of prealbumin could result in a different rate of glomerular clearance. Alternatively a different tubular reabsorptive mechanism could be responsible. In addition prealbumin binds thyroxine (Tata, 1959), and could therefore be a significantly larger molecule than the pure protein.

Fibrinogen is an exception to the general relatively spherical shape of plasma proteins. Viscosity data indicate that it is a long thin molecule, about 475 Å long and 60 Å wide (Hall and Slayter, 1959). It is therefore not surprising that the clearances are slightly lower than expected, since

the effective area which the molecule can occupy is significantly greater than that of a more spherical molecule of the same molecular weight. In addition to the unusual shape, fibrinogen clearances are also difficult to interpret in view of the formation of degradation products, which can react antigenically (Nilehn and Hilsson, 1964). It has been suggested that fibrinogen can deposit on the basement membrane (Vassalli, Morris and McCluskey, 1963; Vassalli, Simon and Rouiller, 1963); this may also play a part in contributing to the low clearance values.

Enzymes

The urinary concentrations of three of the four enzymes studied were higher than normal, and had a correlation with total urinary protein, suggesting an abnormal renal permeability. However only one enzyme, lactic dehydrogenase, showed any degree of correlation with selectivity values.

The enzymes not present in kidney tissue had relatively higher clearances than those present in kidney tissue. Any contribution of renal lactic dehydrogenase or glutamic oxaloacetic transaminase to the urinary level was therefore not demonstrated by the comparison. Although multiple forms of glutamic oxaloacetic transaminase (Martinez-Carrion et al., 1965), pepsinogen (Hanley, Boyer and Naughton, 1966), amylase (Norby, 1964), and lactic dehydrogenase (Appella and Markert, 1961) have been described, there is no evidence that these represent different molecular weights.

Levels of some urinary enzymes in renal disease have been studied by several workers (Kemp and Laursen, 1960; Coltori et al., 1963; Szasz et al., 1965) and found to be elevated, but there has only been one paper relating the clearance of an enzyme to the clearance of albumin (Crockson, 1961). Crockson found the clearance of lactic dehydrogenase to correlate with the albumin clearance. In the present study lactic dehydrogenase clearances

correlated with selectivity in 10 of 14 cases. It is of interest that in three patients, in whom there was no correlation, the proteinuria was very unselective. Since the unselectivity was associated with severe renal damage, it is possible that in these cases renal lactic dehydrogenase was contributing to the high clearance values.

Enzymes are present in serum and urine in minute amounts when compared to the serum proteins and they are relatively labile compounds. Although, being proteins, they should theoretically have a similar renal transport mechanism to that of the serum proteins, their specialised structure and function may preclude this. Tubular secretion, lack of tubular reabsorption, or contributions from sources other than the kidney may be responsible for the high clearance values.

Dextrans

Although the dextran selectivity values were within the range of protein selectivity values they were, with a few exceptions, significantly higher. This difference is unlikely to be methodological; the errors and limitations of the methods have been fully assessed and discussed (2.4.2). Preliminary studies on renal permeability to polyvinylpyrollidone (PVP), in 7 patients with renal disease, have been carried out by Hulme and Hardwicke (1966). The results are not directly comparable, as the molecular weight range studied was lower than in this series. However, it is of interest to note that although the permeability to PVP and protein was similar, in 6 of the 7 cases the PVP slope was higher, that is, the "PVP selectivity" was higher.

Dextran molecules are longer and thinner than plasma protein molecules (Gronwall, 1957). However, a comparison of the normal renal permeability to dextran and protein (over molecular weight range under

70,000) suggests that there is little difference in the renal handling of these macromolecules (Hardwicke and Soothill, 1961). Moreover, since Sephadex is a more accurate measure of molecular radius than molecular weight (Ackers, 1964), by estimating both protein and dextran selectivities by the use of gel filtration the molecular size distribution is being similarly assessed, and indices of selectivity are therefore directly comparable.

Dextran in vivo has been reported to undergo chemical changes. Administration of C^{14} dextran results in production of expired labelled CO_2 and excreted labelled glucose (Cargill and Bruner, 1951; Gray, Sitteri and Pulaski, 1951), but further work has indicated that the metabolism only occurs after dextran has been taken up by the tissues (Terry et al., 1953; Gray, 1953). Although, theoretically, the breakdown of the dextran molecules in vivo could result in a higher selectivity value, the short time intervals involved in the present experiments make this very unlikely.

The enzyme probably responsible for the metabolism of dextran in vivo is present in the kidney (Rosenfeld and Lukomskaya, 1957; Rosenfeld and Saienko, 1964), and could therefore be excreted in the urine. However, significant changes in dextran selectivity were not observed until the urine had been stored for 2 months, moreover the dextran splitting enzyme has a pH optimum of 4.8. Therefore high selectivity values were unlikely to be due to degradation of dextran.

There are no significant glomerular changes after administration of dextran (James and Ashworth, 1961) and although tubular changes have been described (Zettergren, 1962; Maunsbach, Madden and Latta, 1962) there is no evidence to show that the renal ultrastructure is significantly affected. Renal function has been reported to change in glomerulonephritis after administration of dextran, with a rise in glomerular filtration rate of

20 ml./min. (Klutsch, Heidland and Kammerer, 1965). A slight rise in creatinine clearance was also noted in the present experiments, and the dextran selectivity values showed a minimal rise. This was probably a result of the expansion of the plasma volume, which has been noted to cause an increase in albumin clearance (James, Gordeilo and Metcalf, 1956; Malmendier et al., 1960). Both these groups concluded the glomerular permeability was increased. However in two patients protein selectivity was estimated before and during administration of dextran and there was no significant difference in the values. It therefore seems unlikely that alterations in renal function or structure were entirely responsible for the high dextran selectivity values.

The possibility remains that some aspect of the difference in tubular treatment of proteins and dextrans is responsible for the differences in selectivity values. Preferential tubular reabsorption of certain proteins is probably not responsible. Although there is some histological evidence that dextran is reabsorbed by the tubules (James and Ashworth, 1961), experimental work on dogs has demonstrated that tubular interference is insignificant and escapes detection by common clearance techniques (Brewer, 1951; Wallenius, 1954). Since there is evidence that normal, as well as functionally abnormal, glomeruli filter macromolecules (1.2.), every glomerulus capable of filtration can presumably filter dextran. The dextran in the urine therefore represents unchanged the contribution of the filtrates from all the functioning glomeruli. Proteins, on the other hand, are subject to tubular reabsorption and the protein in the filtrate of a normal glomerulus is almost entirely reabsorbed. Only the protein in the filtrate of a functionally abnormal glomerulus is sufficient to swamp the tubular reabsorptive mechanism. Protein in the urine therefore represents

the contribution of the filtrates only from abnormal glomeruli. It follows that selectivity values of dextran will reflect the function of all the glomeruli, while those of protein will reflect only the function of the abnormal glomeruli.

If the renal lesion is homogeneous, and all the glomeruli are similarly abnormal in function, then protein and dextran selectivity values should theoretically agree. If the renal lesion is patchy, however, with some glomeruli more affected than others, then it is reasonable to assume the permeability will also vary. In this situation dextran and protein selectivity values might not agree. Although in the case of dextran selectivity values the contribution of the more abnormal glomerulus will be greater, the selectivity obtained will nevertheless reflect every glomerulus capable of filtration, and an overall picture of glomerular permeability will be obtained. As far as proteins are concerned, the tubular reabsorption will magnify the contribution of the glomerulus filtering the greatest amount of protein, and will eliminate the contribution of the glomerulus filtering an amount below the tubular reabsorptive maximum. This will result in a protein selectivity value which reflects, presumably, the more abnormal glomeruli. Protein selectivities will therefore be lower than dextran selectivities in these cases. A comparison of the values of dextran and protein selectivity would, on this hypothesis, indicate the degree of homogeneity of the renal lesion.

When the results are examined in this light it would seem, from the small amount of data obtained, that patients with minimal lesion glomerulonephritis and membranous glomerulonephritis should have the most homogeneous glomerular lesion, and that patients with proliferative glomerulonephritis may have differing degrees of inhomogeneity. This will be discussed later

(4.1.3). The very high dextran selectivity values obtained in normal subjects could also indicate a high degree of inhomogeneity, although doubts about the protein results have already been expressed. At all events, the overall permeability of the normal glomeruli is obviously highly selective. The patients with minimal proteinuria, including those with acute ischaemic renal failure, myelomatosis and the patient who recovered from minimal lesion glomerulonephritis also had very high dextran selectivities, and therefore, overall, a highly selective renal permeability, which again could theoretically be interpreted as a lack of homogeneity. It is of interest to note that the PVP and protein results of Hardwicke and Hulme (1966) are most similar in minimal lesion glomerulonephritis, and show greatest disparity in proliferative glomerulonephritis and myelomatosis. The dextran results will be discussed further in the section dealing with glomerular ultrastructure (4.1.3).

The change in the slope of dextran selectivity towards lower molecular weights may represent a genuine change in permeability. An alternative, and more likely, explanation is that it was due to the poorer resolution on Sephadex and the more rapidly changing plasma and urine patterns at the lower molecular weights. Values of dextran clearance in relation to the creatinine clearance were similar to those of Hulme and Hardwicke (1966).

4.1.3. SELECTIVITY, DIAGNOSIS, AND GLOMERULAR ULTRASTRUCTURE

Diagnosis

Although selectivity values cannot accurately distinguish between different forms of renal disease, certain ranges of values have been shown to be specific for certain diseases. However, the suggestion made by Blainey et al. (1960) that membranous glomerulonephritis was more selective than proliferative glomerulonephritis, and that the diseases could be distinguished on this basis, has not been confirmed. Subsequently Hardwicke and Soothill (1961) have mentioned that in some cases of proliferative glomerulonephritis they found a highly selective pattern. In this discussion, Hardwicke also mentions variable selectivities in amyloid disease and diabetic glomerulosclerosis and low selectivities in lupus nephritis. In the present study, patients with amyloid disease were almost all selective and patients with lupus nephritis and diabetic glomerulosclerosis generally had high or intermediate values. Joachim et al. (1964) also found high selectivity values in amyloid disease, but their patients with lupus nephritis had very variable values and their patients with membranous glomerulonephritis were, on the whole, more selective than these of the present series. This may, however, be due to differences in histological classification; electron microscopy was not used by Joachim et al. and it is interesting to note that the distribution between membranous glomerulonephritis and proliferative glomerulonephritis was very different from that in this study. Differences in classification may also account for the results of Hitzig, Aurricchio and Benninger (1965), which were not entirely in agreement with those of the present study. These authors classified selectivity into three types, but found considerable overlap, particularly between minimal lesion glomerulonephritis and membranous glomerulonephritis. Of the 48 patients studied,

however, diagnosis was supplemented by biopsy or autopsy specimens in only 27 cases. From the experience of the present series electron microscopic examination is essential to the histological diagnosis of minimal lesion glomerulonephritis.

Patients with postural proteinuria and the one patient with exercise proteinuria were found to have an unselective pattern, which confirms the work of Rowe and Soothill (1961b). No other studies of selectivity values have been recorded in the literature, with the exception of the study of children by Cameron and White (1965). These authors found very high selectivity values associated with minimal lesion glomerulonephritis, as found in both adults and children in the present study.

The correlation of selectivity and diagnosis is important, since it demonstrates selectivity could be of value in differential diagnosis. The opinion, held by many, that minimal lesion glomerulonephritis is a mild form of membranous glomerulonephritis will be questioned later, but the present selectivity studies have demonstrated that the renal permeability in these diseases is quite different. The present series also shows that a high selectivity value may be confirmatory for amyloid disease, and a low selectivity value for acute ischaemic renal failure and postural proteinuria. Of the remaining disease groups that were studied, a value of -k of over 2.0 was rarely seen in membranous glomerulonephritis and never in mixed membranous and proliferative glomerulonephritis, while values of -k of under 1.30 were only seen in membranous glomerulonephritis, proliferative glomerulonephritis and rarely in lupus nephritis.

Data on patients in the miscellaneous group is as yet inadequate for diagnostic use, but it indicates that a high selectivity is associated with potassium depletion and hypertension, an intermediate selectivity with renal vein thrombosis and a low selectivity with toxaemia of pregnancy.

The comparison of selectivity values in toxaemia of pregnancy with those of accidental haemorrhage was carried out in view of doubt about the inter-relationship of these diseases (Paterson, 1966). However further studies with renal biopsy are required before any significant conclusions can be drawn.

Glomerular ultrastructure

The structure of the glomerular capillaries in proteinuria has been considered by many workers, and it has been concluded that no single specific lesion is responsible for proteinuria, although some defect in the basement membrane is probably of prime importance (Spiro, 1959; Churg et al., 1962). Some studies have been carried out in an attempt to relate ultrastructural and functional changes (Galan and Maso, 1957; Muehreke et al., 1957; Movat, Steiner and Slater, 1961; Drummond et al., 1966), but only three studies relating to the ultrastructural appearances to renal permeability have been published (Blainey et al., 1960; Joachim et al., 1964; Hitzig et al., 1965). No selectivity studies involving electron as well as light microscopy have been described.

In the present study a variety of glomerular abnormalities were seen. A general consideration of the entire group of patients showed an interesting spectrum, with increasing glomerular damage associated with an increasing lack of selectivity. Cameron and White (1965) have suggested that there is a natural upper limit to selectivity values, and that this limit is approached in minimal lesion glomerulonephritis, where glomerular changes are very slight. If selectivity is assumed to reflect glomerular permeability, this would suggest that normal glomeruli are highly selective and that only as the degree of glomerular damage increases can the selectivity value decrease. Although the correlation of degree of glomerular damage and

selectivity has been demonstrated by Joachim et al. (1964) and in the present study, the protein selectivity in subjects with normal or almost normal glomeruli was very low. However, the dextran selectivity was very high and since dextran selectivity is theoretically a more reliable index of glomerular permeability, particularly at low levels of protein excretion, this provides strong evidence of a highly selective normal glomerulus. Normal glomerular permeability will be discussed more fully later (4.2.1). On this hypothesis, the index of selectivity, during significant proteinuria, becomes a potentially valuable estimate of the degree of glomerular damage.

In addition to the overall correlation of selectivity and glomerular ultrastructure, the significance of the changes in the main filtration barrier, the basement membrane, was well demonstrated in this study. In membranous glomerulonephritis the progression of the disease and the loss of selectivity were both associated primarily with basement membrane changes. Dark material, or osmiophilic deposits in the basement membrane were associated with only the lowest selectivity values of lupus nephritis. A similar dark basement membrane material was also associated with the low selectivity values of mixed membranous and proliferative glomerulonephritis, and basement membrane deposits were seen in toxaemia of pregnancy, where selectivity was also low. The glomerular changes of toxaemia are also interesting in view of the striking endothelial changes and normal pedicel structure, the reverse of the situation normally seen.

No basement membrane changes could be seen in minimal lesion glomerulonephritis, with the exception of two patients in whom a slight thickening was noted. Selectivity values in these two patients however were not significantly different from those of the rest of the group. The dextran selectivity estimated in a patient during proteinuria, and when proteinuria

was abolished by steroid therapy, demonstrated that glomerular permeability in minimal lesion glomerulonephritis is similar to that of normal subjects; although the slight rise in dextran selectivity on recovery suggested the permeability may be marginally increased during the disease. This apparent increase in glomerular permeability could be due to an invisible basement membrane lesion, but the possibility of a lack of tubular reabsorption can not be excluded. A combination of altered glomerular permeability and lack of tubular reabsorption has been considered by some workers to be the origin of proteinuria (1.2). In minimal lesion glomerulonephritis, where glomerular changes are minimal and are possibly the result rather than the cause of proteinuria, lack of tubular reabsorption could be a major factor. In addition, it cannot be excluded as a contributory factor in any disease with significant glomerular involvement.

The presumed relationship of minimal lesion and membranous glomerulonephritis has been mentioned earlier. However, ultrastructural studies have demonstrated a distinct difference between the diseases, and no patient in this series with minimal lesion glomerulonephritis has been observed to progress to membranous glomerulonephritis. A histological similarity between some cases of mild proliferative glomerulonephritis and minimal lesion glomerulonephritis, however, has been noted. The selectivity results entirely support this view.

Although selectivity can generally be considered in terms of ultrastructure, in some cases the results must be interpreted with more caution. The high degree of selectivity in amyloid disease was associated with gross basement membrane changes. It is possible that the fibrillar structure in this disease can act as a highly selective filter, in spite of the effect on renal function; in these cases the creatinine clearance was often

markedly reduced. In proliferative glomerulonephritis the selectivity values were difficult to interpret in terms of ultrastructure, as there was no obvious correlation between the two. Possibly some additional factor or factors, at the sub-electron microscopical level, are affecting the glomerular permeability. Alternatively the inhomogeneity of the renal lesion, which was noted in some biopsy tissue in these cases, could be relevant.

The significance of the dextran results has already been discussed (4.1.2) and it is of great interest that in proliferative glomerulonephritis, in addition to the lack of correlation between protein selectivity and ultrastructure, there was also a lack of correlation between protein and dextran selectivity. In proliferative glomerulonephritis renal function also bore a poor correlation to protein selectivity. These findings can all be interpreted in terms of inhomogeneity of the renal lesion. On the other hand, the ultrastructural studies suggested that membranous and minimal lesion glomerulonephritis had a much more homogeneous type of lesion, and this was confirmed by the correlation of protein selectivity values with both the ultrastructure and dextran selectivity. Further dextran studies are required however before there is adequate support for this concept.

The selectivity values in chronic renal failure suggest that the lower limit of selectivity, when glomeruli become grossly diseased, is a value of -k of about 1.0. Patients with advanced membranous glomerulonephritis and the patient with pyelonephritis both had a low selectivity associated with gross glomerular changes. However, in the patients with postural proteinuria and acute ischaemic renal failure the paradoxical situation of even lower selectivity values and very minimal glomerular damagewas found.

The mechanism and origin of proteinuria in these diseases is uncertain, but a consideration of ultrastructure and selectivity may prove helpful in interpretation.

The histological similarity of biopsy tissue from patients with postural proteinuria and minimal lesion glomerulonephritis might suggest that the origins and mechanisms of the proteinuria were similar. However, the very different selectivity values demonstrate that the renal, if not glomerular, permeability is very different. Studies on postural proteinuria have indicated that the condition may progress to continuous proteinuria, and be associated with a significant renal lesion (Robinson et al., 1961 a and b; King, 1959), but in these series patients were not subjected to renal biopsy when they were first seen. Postural proteinuria had been suggested to be the result of increased transfer of plasma proteins through the glomerulus (Slater, O'Doherty and Wolfe, 1960), and if the normal kidney has a few 'leaky' glomeruli this mechanism is a possibility. A lack of tubular reabsorption in this situation could also be a factor. Lowgren (1955), however, considers that postural proteinuria is post-renal, and is derived from the renal lymphatic system. The mechanism of postural proteinuria is therefore uncertain. However, it is possible that it does not arise from a significant glomerular abnormality, and dextran studies in these cases may confirm this view.

In acute ischaemic renal failure the main histological lesion is seen in the tubules, and impaired tubular reabsorption could theoretically give rise to significant proteinuria. The aminoaciduria in acute tubular necrosis has been thought to be due to lack of tubular reabsorption (Emslie-Smith et al., 1956), and the concept is further supported by the work of Goodman and Baxter (1956), which demonstrated decreased tubular reabsorption

of protein and proteinuria in rats with minimal glomerular damage and marked tubular necrosis, induced by uranyl acetate. In view of the marked tubular changes, a leak of plasma protein directly into the tubular lumen is also theoretically possible and would give rise to a proteinuria of zero selectivity. Assuming normal glomerular permeability to be selective, a combination of impaired tubular reabsorption of protein, filtered normally, and a leak of plasma protein could result in a proteinuria of an unselective pattern. The rise in selectivity demonstrated after infusion of albumin supports this view. Alternatively, a very patchy renal lesion with a few grossly damaged glomeruli (which could also be the situation in normal proteinuria (4.2.1)), combined with lack of tubular reabsorption could produce the same results. The dextran results in these cases confirmed that the glomerular permeability is overall of a normal highly selective pattern.

Lack of tubular reabsorption in patients with potassium depletion and hypertension may also be the cause of the proteinuria. An increase in proteinuria in potassium depleted rats has been demonstrated, and was thought to be due to a decrease in tubular reabsorption of protein (Morrison and Gardner, 1963). If this mechanism operated in potassium depleted patients, who have high selectivity values, a selective normal glomerulus is again implied.

Of the remaining patients in the miscellaneous group, those with multiple myeloma, exercise haematuria and proteinuria of unknown origin had selectivity values which were difficult to interpret in terms of ultrastructure, although, significantly, basement membrane changes were seen in two patients with proteinuria of unknown origin. In exercise haematuria the possibility of mechanisms similar to those in postural proteinuria must

be considered. Patients with multiple myeloma present an interesting problem, since a gross quantity of small molecular weight Bence-Jones protein is excreted and this might be expected to swamp the normal tubular reabsorption. In the one patient studied the amount of serum protein excreted was surprisingly low, indicating that the Bence-Jones protein may, to a certain extent, have a separate tubular reabsorptive mechanism. In view of the almost normal glomerular appearance, the pattern of the resulting proteinuria derived from the serum proteins might be predicted as selective. Although protein selectivity was low, the dextran selectivity was high, confirming the glomerular permeability was selective and that the origin of the proteinuria was perhaps again due to a 'leak' of plasma protein.

The two patients with renal vein thrombosis had glomerular appearances similar to those seen in proliferative glomerulonephritis and membranous glomerulonephritis, and selectivity values compatible with this. The two patients with post-partum renal disease showed unusual glomerular changes, which have never been described before, and are difficult to relate to selectivity values.

4.2. GENERAL DISCUSSION.

4.2.1. IMPLICATIONS OF SELECTIVITY STUDIES

Proteinuria can theoretically arise in a number of ways. It has been assumed, when discussing the results, that protein selectivity is a measure of renal permeability. However, if this is the case certain criteria must be satisfied.

(1) Permeability can be assessed by measurement of the ratio of the concentration of a substance on either side of a hypothetical renal membrane.

(2) The urine proteins are derived unchanged, directly and solely by renal transport from the serum.

(3) Renal tissue, the urinary tract and the urine have no metabolic effect on the serum proteins.

(4) Concentration of the urine proteins and their assay procedures have no physical or chemical effects, which would change the apparent concentration, or molecular weight of the protein.

(5) The molecular weights of the proteins correspond to their effective molecular size.

(6) The relationship of logarithm of renal clearance of protein and logarithm of molecular weight of protein is consistently linear.

The permeability of a membrane (1) should theoretically be defined in terms of volume, or mass, of substance passing through unit area, in unit time, under the influence of unit hydrostatic, or unit osmotic pressure (Landis, 1946). The situation is further complicated in the kidney by the complexity of the filtration and reabsorption mechanism. However if the kidney can be assumed, for the purposes of assessing selectivity, to be a

single membrane, then the ratio of the concentration of protein on either side of the membrane is an acceptable simplification of permeability.

In renal disease criterion (2) is almost certainly satisfied. However, the evidence is circumstantial since the serum origin has never been confirmed directly. Excessive catabolism of serum proteins has been reported to occur, although not invariably, in renal disease (Gitlin, Janeway and Farr, 1956; Kaitz, 1959; Anderson, 1962; Nussle and Royer, 1963), and higher levels of amino acids have been found in the renal vein than in arterial blood (Eliasch et al., 1955). However, there is no evidence that circulating fragments of serum proteins contribute significantly to the urine proteins, with the possible exception of the proteinuria of cadmium poisoning (Squire, Hardwicke and Soothill, 1962). In addition to the evidence presented in the introduction (1.2), the identity of serum and urine proteins from nephrotic patients was confirmed in the present study, by the correlation of results of immunological and gel filtration experiments.

The identity of serum and urine proteins also contributes to satisfy criterion (3) and King and Boyce (1963), in a discussion of the source and metabolism of serum derived proteins in the urine, also provide support for this point. There are suggestions that renal tissue contains active proteolytic enzymes (Harms et al., 1962) and that the kidney is a site of albumin catabolism (Eliasch et al., 1955; Hughes, 1957), but these effects are probably of little significance in the proteinuria of renal disease.

In a recent paper, Miyasato and Pollak (1966) have stated that they found a loss or decrease of high molecular weight protein as a result of concentration procedures; this concerns point (4). The principal concentration procedure for urinary proteins in this study was carefully checked in several ways, and no significant loss or change in properties of the

proteins was found. Although β-lipoprotein could not always be detected in the urine, there was no evidence to suggest that the concentration procedure adopted was responsible for its absence. Results using ultrafiltration agreed with those using polyethylene glycol, and many other workers have found both these methods to be reliable (Grant, 1957; Berggard, 1961a, 1961b; Howe, Groom and Carter, 1964). The assay procedures in this study were reliable; immunological methods of estimating protein are generally assumed to reflect the total amount of protein present, and gel filtration has not been observed to have any polymerising or degradative effects on protein.

Sephadex G 200 separates protein on the basis of molecular radius rather than molecular weight, so the question of (5) is only relevant for the immunodiffusion studies. Oncley, Scatchard and Brown (1947) have demonstrated that, with the exception of γ-globulin, serum proteins approximate to a spherical shape and, although the structure and amino-acid composition of proteins may vary, the relationship between molecular weight and molecular size generally holds good (Sobotka, 1955).

The linear relationship of logarithm of renal clearance of protein and logarithm of molecular weight of protein, point (6), has recently been questioned by Barcelo and Pollak (1966). However, in this study quite different results were obtained. The relationship always approximated to a linear one, whether it was measured immunologically or by gel filtration, and the degree of linearity was consistently highly significant statistically. This is in agreement with the work of Blainey et al. (1960) and Joachim et al., (1964) and Hitzig et al. (1965). Occasionally a slight 'dog-leg' profile was found when the γ-globulin clearance was consistently high or low, but this was rare and may have been due to the unusual shape of this

molecule. The molecular weight of transferrin has been more recently estimated as 68,000 (Charlwood, 1963), which may account for the high transferrin clearances of over 100%, which were sometimes observed. Only two out of eleven proteins, measured immunologically, and some enzymes were found to deviate from linearity, and with good theoretical reasons.

The above reasoning suggests that selectivity of proteinuria in renal disease can be accepted as an accurate index of renal permeability. However, if selectivity is to be accepted as an index of glomerular permeability further assumptions must be made:-

(7) The glomerulus is the source of the proteinuria.

(8) Tubular reabsorption of protein is competitive.

(9) Every glomerulus is involved in making a contribution to the proteinuria.

The evidence to support (7) has been presented in the introduction and significant glomerular changes, with some exceptions, were seen in association with proteinuria in this study. Although lack of tubular reabsorption has been cited as a factor in some forms of renal disease, this does not invalidate the glomerulus as the source of the proteinuria.

Competitive tubular reabsorption of protein, point (8), in proteinuria was demonstrated by Hardwicke and Squire (1955); the present study has given no indication that this is not the case. Selectivity studies in themselves lend support to the concept, since the stability of the values when total protein excretion changes suggests a competitive type of tubular reabsorption.

It is possible to comment on point (9) from histological evidence. Renal biopsy, as well as autopsy material, have shown that some types of renal lesions are more homogeneous than others. The studies using dextran

also strongly suggested that this is the case, and the fact that histological predictions of homogeneity correlate with dextran findings adds support to the concept. The interpretations of the protein selectivity studies in this situation have already been discussed.

It is therefore suggested that during proteinuria an index of glomerular permeability can be obtained from selectivity studies, although protein studies may reflect only the more abnormal glomeruli. More dextran studies are required, but it is suggested that the results reflect the permeability of all the glomeruli, and that comparison of the dextran and protein results may give an indication of the degree of imhomogeneity of the glomerular lesion.

In normal subjects and patients with trace or normal amounts of protein in the urine, selectivity studies must be interpreted with more caution. Gel filtration results have demonstrated that condition (2) is certainly not always satisfied, and in addition the validity of (3) and (5) may also be in doubt. An increased amount of protein in the urine, which is not of serum origin, is also found in other conditions (Boyce, Garvey and Norfleet, 1955; Di Ferrante, 1957). The source of the additional proteins in the urine is thought to be the urinary tract (Grant, 1957, 1959), but a convincing demonstration of their origin has not been carried out. Other possible sources include the kidney and the genital system. Uromucoid is raised in pyelonephritis, but not acute cystitis (Boyce and King, 1960), suggesting it is renal in origin, but there is no evidence of its presence in renal tissue (Boyce, King and Fielden, 1961). Analyses of non-dialysable solids in male and female urine have been reported to be similar (Boyce et al., 1958). However, Anderson and Maclagan (1955) found a significantly higher amount of mucoprotein in male urine than in female urine, suggesting

that a significant contribution from the genital system should not be excluded.

Although immunodiffusion can be used to estimate selectivity at lower levels of proteinuria than gel filtration, the validity of this method also becomes doubtful in normal subjects, since the source of the unselective normal pattern is uncertain. The kidney or urinary tract could contribute proteins to the urine which are immunologically similar to serum proteins. Alternatively, the proteins may genuinely be derived from the serum, either by selective filtration of degraded proteins, unselective filtration of unchanged proteins or by leakage of protein from the tubules. However, tubular leakage is unlikely, and degradation of protein may not be significant. The fact that the normal Υ-globulin clearances correlate less well with molecular weight than the clearances of other proteins suggests that Υ-globulin may be the only significantly degraded serum molecule in normal urine. Although few unchanged serum proteins were detected in normal urine using gel filtration and immunoelectrophoretic techniques, they may have been masked by the large amounts of foreign protein present.

The possibility remains that normal glomerular filtration is the source of the unselective proteinuria and if selectivity reflects glomerular permeability criteria (8) and (9) must be examined. Little is known about the normal tubular reabsorptive mechanism (8). By analogy with renal disease it may be assumed competitive, but the possibility exists that the competitive mechanism may only be displayed during disease. The dextran selectivity suggests that overall the normal glomerulus is highly selective. Therefore if the urinary proteins are derived unchanged, by normal glomerular filtration and competitive reabsorption, very few glomeruli can be involved (9). No glomeruli of a degree of abnormality compatible with the lack of selectivity

of normal proteinuria have ever been seen in normal renal tissue, but the statistical chances of this happening are small.

In summary, the source of the proteins of serum origin in normal urine therefore remains uncertain, although theoretically they could be derived in a number of ways, in particular from a few 'leaky' glomeruli. Protein studies therefore are of little value in estimation of overall normal glomerular permeability. Dextran studies, however, indicate a highly selective mechanism.

Glomerular permeability has been described in terms of 'pore size' (Hardwicke and Soothill, 1961; Ozawa and Yamuchi, 1963). These pores can only be regarded as functional pores, since breaks or pathways in the basement membrane have never been described by any electron microscopist. They provide a useful concept, however, for interpretation of the mechanism of glomerular permeability. In renal disease the size of the glomerular pores can be thought of as relating to both the ultrastructural changes and the selectivity of the proteinuria. Numerous small pores, associated with little glomerular damage and perhaps similar in size to normal pores, would give rise to a selective proteinuria, as in minimal lesion glomerulonephritis. Enlarged pores, associated with gross glomerular damage, would give rise to an unselective proteinuria, as in advanced membranous glomerulonephritis or chronic renal failure, the degree of lack of selectivity being a reflection of the increasing pore size. A change in the number, rather than the characteristics, of the pores contributing to the proteinuria could fully account for the stability of selectivity, which has been observed while proteinuria is diminishing. The question of different populations in pore size has been raised by Mayerson et al. (1960). The data obtained by Gregoire et al. (1958), following albumin infusions, suggest that at least two populations are present in nephrosis, and if the clearance plots are

considered as curves rather than two straight lines, then a gradation of nephrons contributing to the proteinuria must be postulated. The dextran results in this study also indicated an inhomogeneity of the renal lesion, which would correspond to a distribution of pore sizes.

The comparison of selectivity and ultrastructure has indicated that the basement membrane is of prime importance in controlling glomerular permeability; the size of the functional pores can therefore be related to the degree of disorder of the basement membrane. This was well demonstrated in membranous glomerulonephritis. However, selectivity, while correlating overall with ultrastructure, demonstrated that in some diseases additional factors are controlling glomerular permeability. The variation in pore size, suggested by the dextran results, particularly in proliferative glomerulonephritis, may be one such factor. The type of renal damage, rather than the degree and extent, may be another; this was well demonstrated in amyloid disease. The etiology of the disease process has not been considered in this study, but could also be a factor, and it may be significant that glomerular ultrastructure and selectivity correlated well in diseases with a single etiology, lupus nephritis and diabetic glomerulosclerosis. Deposition of γ-globulin on the basement membrane has been suggested to relate to an immunological response and hence to etiology (Freedman, Peters and Kark, 1960; Michael et al., 1964; Okuda et al., 1965). However, significant deficiencies of γ-globulin in the urine were not demonstrated by the clearance techniques in any of the renal diseases studied.

The lack of tubular reabsorption as a contributory factor in proteinuria has been discussed and concluded to be of possible relevance, particularly in diseases where there was no significant abnormality in the glomerulus. This included patients with minimal lesion glomerulonephritis,

potassium depletion, acute ischaemic renal failure and to a lesser extent postural proteinuria, exercise proteinuria, and multiple myeloma. The origin of the minimal proteinuria in acute ischaemic renal failure, postural proteinuria, exercise proteinuria, and multiple myeloma raises particular problems in view of the unselective pattern and apparently normal pore size. The possible mechanism, including abnormal tubular function and a markedly inhomogeneous renal lesion (perhaps analogous to normal glomerular permeability) have already been discussed.

Glomerular permeability to macromolecules which are smaller than the serum proteins has not been investigated in this study. In normal subjects, experiments using dextran and polyvinyl pyrollidone have demonstrated high selectivities to molecules with effective radii between 25 and 60 Å (Arturson and Wallenius, 1964b; Hulme and Hardwicke, 1966). In renal disease, however, although the permeability is increased at high molecular weights, there is impaired permeability at lower molecular weights (Ozawa and Yamuchi, 1963; Hulme and Hardwicke, 1966). Linearity of the relationship between renal clearance and molecular weight on a log-log scale has been assumed, and approximated to, in all the present selectivity studies, but it is unlikely to be strictly valid. An examination of the relationship over a very wide range of molecular size might reveal a curvilinear pattern, corresponding to a normal or log-normal distribution of pore size. Such a distribution would be shifted to higher values in renal disease with glomerular impairment.

A generalised increase in capillary permeability in the nephrotic syndrome may be questioned, in view of the marked oedema formation. The protein distribution in oedema fluid has been noted to be similar to that of the urine in renal disease (Freeman and Joekes, 1957). Becker (1964) has demonstrated higher salivary concentrations of some plasma proteins in

patients with the nephrotic syndrome than in normal subjects. Similar losses of protein at several sites may account, in part, for the excessive catabolism of protein that has been noted in some patients with the nephrotic syndrome.

4.2.2. VALUE OF SELECTIVITY DETERMINATIONS

Indices of protein selectivity in renal disease have been shown to be, on the whole, extremely stable in spite of large variations in protein excretion and renal function. Selectivity, with certain qualifications, can reflect glomerular permeability and the constancy of the values therefore makes the estimation particularly valuable, since glomerular permeability can be assessed regardless of the clinical or biochemical condition of the patient.

Indices of protein selectivity are of potential value in differential diagnosis. Specific glomerular changes are not always confined to one disease group, and therefore selectivity cannot be expected to correlate perfectly with diagnosis. However, the results have indicated that selectivity estimations can have a valuable contribution; for example in distinguishing minimal lesion glomerulonephritis from membranous glomerulonephritis, or postural proteinuria from some other type of renal disease with glomerular involvement.

Glomerular ultrastructure has also been shown to correlate with protein selectivity. In some cases the selectivity can assess the degree of disorder of the basement membrane, while in others the overall severity of the disease is reflected. This in turn relates to the prognosis, as a poor prognosis is always associated with gross glomerular damage and therefore with an unselective proteinuria.

The greatest practical value of selectivity estimations lies almost certainly in the ability to predict responsiveness to steroid treatment. Of the patients with selectivity values of over 1.9 who were given steroids, 78% responded to the treatment, and this included patients with several

different types of renal disease. On the other hand, only 4% of the patients with selectivity values of under 1.9 who were treated with steroids responded.

At present, indices of selectivity are becoming a valuable adjunct to the histological and ultrastructural studies of the renal lesion. In cases where a renal biopsy, for some reason, is unobtainable or unsuccessful, the selectivity of the proteinuria can provide valuable information. The degree of ultrastructural abnormality, prognosis, in some cases diagnosis, and above all, responsiveness to steroid therapy may be predicted. Indices of dextran selectivity may also be of value in assessing the degree of homogeneity of the renal lesion, and in cases of minimal proteinuria the dextran selectivity will give a more reliable estimate of the glomerular permeability.

The potential value of selectivity determinations suggests an estimate should be part of the assessment of every patient presenting with proteinuria. At present methods of estimating selectivity are somewhat tedious for routine purposes. A modification of the immunological method, using only two proteins may be practicable and sufficiently accurate. The best choice of proteins would be albumin or transferrin and α_2-macroglobulin. β-Lipoprotein was not always detected in the urine and clearances of γ-globulin were sometimes found to deviate slightly from the linear relationship; the suggested method of Cameron and Blandford (1966) would therefore not be suitable on the basis of the present study. Alternatively, estimation of selectivity by thin layer gel filtration on Sephadex G 200 (Morris, 1964) may be feasible.

More estimations of dextran selectivity are required before the value of this approach can be fully assessed. The recent method, enabling dextran to be labelled with radioactive iodine (Ricketts, 1966), could be useful in simplifying selectivity estimations. Smaller doses of dextran

could be given, avoiding any plasma expansion. It would be possible to investigate glomerular permeability over a much wider molecular weight range by this technique. It would also be interesting to compare dextran and polyvinyl pyrollidone clearances.

Further studies on permeability and ultrastructure are required before the mechanism of glomerular filtration of macromolecules is fully understood. Studies combining clearance estimations with a direct view of the transport at the ultrastructural level might prove valuable. A high molecular weight labelled compound, which could be seen on electron microscopy, such as Imferon (Ricketts et al., 1965) would be suitable. The degree of glomerular disorganisation might also be reflected by abnormal amounts in the urine of some basement membrane-type material; for example carbohydrates or hydroxyproline (Dische et al., 1965). In addition, more estimations of selectivity, to proteins and to other macromolecules, may reveal further information concerning the glomerular permeability to macromolecules.

5. SUMMARY

Renal permeability to macromolecules has been studied in 130 patients and some normal subjects. The conclusions can be summarised as follows:-

1. The linear relationship between renal clearance and molecular weight of protein on a log-log scale, which was first reported by Blainey et al. (1960) has been confirmed. The slope of the line has been used as an index of "selectivity".
2. Indices of protein selectivity were determined by an immunodiffusion method. Clearances of individual proteins, of known molecular weight, were estimated by measuring the amount of precipitation after serum and urine had been allowed to react with specific antisera. Eleven proteins were tested and nine were found to be suitable for estimating selectivity; values were generally calculated from the clearances of five proteins. Immunoelectrophoresis was also used to determine selectivity in some cases, by comparing both the number and molecular weights of the proteins identified in serum and in urine.
3. A method has been developed for estimating selectivity, by determining the molecular size distribution of serum and urine proteins by gel filtration on Sephadex G 200.
4. The errors of the immunodiffusion and gel filtration methods were assessed. Coefficients of variation for the estimation of selectivity were 4% and 7% respectively. Gel filtration was of doubtful value when the proteinuria was under 1 g./day. Immunodiffusion as a method of estimating selectivity was a more reliable method in minor proteinuria, but was of doubtful value when the proteinuria was under 200 mg./day.

5. Results of the immunological and gel filtration studies correlated well in proteinuria. Since the principles on which immunological and gel filtration methods are based are quite different, the correlation indicated that selectivity is a meaningful estimate of abnormal renal permeability.
6. Renal permeability in disease was found to range from a high degree of selectivity to an almost total lack of selectivity. The indices of protein selectivity were found to be extremely stable over periods of one to two years, in spite of clinical and biochemical variations. Values correlated to a limited extent with renal function and age, but there was no correlation with total protein excretion. Selectivity values showed no significant decrease on infusion of albumin, suggesting confirmation of a competitive tubular reabsorptive mechanism for protein.
7. Renal permeability was not affected by commencement of steroid treatment, nor were there significant changes in selectivity while patients were responding to steroid treatment and proteinuria was diminishing. Selectivity had a striking correlation with the ability to respond to steroid treatment; high values of selectivity were found in 95% of the responsive patients.
8. Renal permeability was examined in relation to diagnosis. Significant differences were found between the selectivity values of some different renal diseases, in particular between the high values of minimal lesion glomerulonephritis and every other type of renal disease, including membranous glomerulonephritis. Patients with amyloid disease also had significantly high selectivity values, and patients with postural proteinuria and acute ischaemic renal failure had significantly low values.

9. Renal permeability was examined in relation to ultrastructure. An association was found between both the degree and type of renal lesion and the selectivity value. In most cases increasing glomerular damage was associated with lower selectivity values. This was well demonstrated by most of the renal diseases studied where there was significant glomerular involvement, and was also seen in individual patients with membranous glomerulonephritis, diabetic glomerulosclerosis and lupus nephritis. The significance of basement membrane changes in causing alterations in renal permeability was noted. Exceptions to the overall correlation between glomerular damage and selectivity were found in amyloid disease, proliferative glomerulonephritis, postural proteinuria and acute ischaemic renal failure, suggesting the type of renal lesion is also of importance in controlling renal permeability.
10. Renal permeability to some enzymes and to dextran was also studied. Enzyme clearances had no correlation with protein selectivity. A method was developed for estimating dextran selectivity, which gave comparable results, over the same molecular weight range, as the protein studies. Indices of dextran selectivity, however, were found in most cases to be significantly higher than indices of protein selectivity. This finding was discussed and it was concluded to reflect an inhomogeneity of the renal lesion. This may be particularly relevant in minimal proteinuria.
11. In normal subjects indices of protein selectivity were low, but for theoretical and experimental reasons were not considered to give an accurate reflection of normal renal permeability. High dextran selectivity values indicated the normal kidney to be extremely selective in the excretion of macromolecules.

12. The selectivity studies were interpreted in terms of glomerular permeability and, following from this, the mechanism of proteinuria in health and disease was discussed. An assessment was made of the value and applications of selectivity estimations in clinical medicine.

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The University Department of Clinical Chemistry carried out most of the estimations of creatinine, urea and total urine protein, and all the estimations of serum protein. Creatinine, urea and total urine protein were occasionally determined by the Renal Unit Laboratory, University Department of Therapeutics. The co-operation of the staff of both laboratories was appreciated.

Skilled technical assistance was given by Miss G. Robb, Miss H. Watt and Miss J. Beskow.

7. APPENDICES

APPENDIX 1. MATERIALS

Material	Details	Supplied by
Immunodiffusion studies		
Agar	Difco Bacto-Agar	Difco Laboratories, Detroit, Michigan.
Antisera	Beringwerke AG, Marburg-Lahn.	Hoechst Pharmaceuticals, Portland House, Stag Place, London, S.W.1.
	Hyland Laboratories	Baxter Laboratories, London Road Trading Estate, High Wycombe, Bucks.
Neuraminidase	crystalline from Vibrio Cholera	Sigma.
Petri dishes	Jener glass	Andermann and Co. Ltd., 87-95 Tooley Street, London, S.E.1.
Template	for Ouchterlony plates	Messrs. Peter Brasshouse, Spring Hill, Birmingham, 18.
Enzyme studies		
$NADH_2$	as the sodium salt	B.D.H.
Dried plasma	for pepsin substrate	Prepared by Blood Transfusion Service, Royal Infirmary, Edinburgh.
Glutamic oxaloacetic transaminase	estimated by a reagent kit, no. 410-50	Sigma
Lintners starch	for amylase estimations	B.D.H.
Dextran studies		
Dextran for infusion:		
Intradex	6% dextran in 0.9% NaCl	Glaxo Laboratories, Greenford
Intraflodex	10% dextran in 5% NaCl	" " "
Dextraven	6% dextran in 0.9% NaCl	Benger Laboratories, Cheshire
Rheomacrodex	10% dextran in 5% NaCl	Pharmacia, Sinclair House, The Avenue, London, W.13.
'Zipettes'	for dispensing solutions	Jencons, Hemel Hempstead, Herts.

Material	Details	Supplied by
Miscellaneous		
Albumin	for infusion	Prepared by the Blood Transfusion Service, Royal Infirmary, Edinburgh, by the Kohn ethanol fractionation method.
Bovine serum albumin	crystalline for microbiuret standards	B.D.H.
Albustix	reagent strips	Ames Company, Stoke Poges, Bucks.
LKB equipment	Ultrafilter-6300A Radi-Rac fraction collector-3400B Immunoelectrophoresis apparatus-6800A	LKB-Producter AB, Stockholm.
Polyethylene glycol, Carbowax 20M		Union Carbide, 8 Grafton Street, London, W.1.
Sephadex G 200		Pharmacia, Sinclair House, The Avenue, London, W.13.

APPENDIX 2. STATISTICAL FORMULAE

from Moroney (1963).

Reproducibility

From N determinations on one sample

standard deviation of mean (SD) = $\sqrt{\frac{\Sigma(y-\bar{y})^2}{N}}$

$\bar{y}$ = mean value

y = individual values

From pairs of determinations on N different samples

standard deviation of mean (SD) = $\sqrt{\frac{\Sigma d^2}{2N}}$

d = difference in values between each pair of observations

Coefficient of variation (CV) = $\frac{SD \times 100}{\bar{y}}$ %

Standard error of the mean = $\frac{SD}{\sqrt{N}}$

Regression lines by the method of least squares

$y = mx + c$

Slope (m) = $\dfrac{\Sigma xy - \frac{\Sigma x \, \Sigma y}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$

Intercept (c) = $\dfrac{\Sigma x \, \Sigma xy - \Sigma y \Sigma x^2}{(\Sigma x)^2 - N \Sigma x^2}$

Correlation coefficient (r) = $\dfrac{\Sigma xy - \frac{\Sigma x \Sigma y}{N}}{\sqrt{[\Sigma x^2 - \frac{(\Sigma x)^2}{N}] \, [\Sigma y^2 - \frac{(\Sigma y)^2}{N}]}}$

Standard error of estimate of y (SE) = $\sqrt{\frac{1}{N}(y - \bar{y})^2 (1 - r^2)}$

Student's t test

$$t = \frac{\text{Difference of means}}{\text{Standard error of difference}}$$

$$\text{sample variance (s)} = \sqrt{\frac{\Sigma x^2}{N} - \bar{x}^2}$$

$\bar{x}$ = sample mean

x = individual values

Best estimate of standard error for the difference of the means of two samples

$$(\sigma) = \sqrt{\left(\frac{n_1 s_1^2 + n_2 s_2^2}{n_1 + n_2 - 2}\right)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

$$t = \frac{\bar{y} - \bar{x}}{\sigma}$$

APPENDIX 3. PUBLICATIONS

Statement in terms of Ph.D. Regulation 13 of the University of Edinburgh.

Part of the work embodied in this thesis has been published as follows:-

Pamela R. MacLean and J. S. Robson (1966).
Unselective proteinuria in acute ischaemic renal failure.
Clin. Sci. 30, 91.

Pamela R. MacLean and J. J. B. Petrie (1966).
A comparison of gel filtration and immunodiffusion in the determination of selectivity of proteinuria.
Clinica chim. Acta, 14, 367.

V. A. Ruckley, M. K. MacDonald, P. R. MacLean and J. S. Robson (1966).
Glomerular ultrastructure and function in postural proteinuria.
Nephron, in press.

8. REFERENCES

ACKERS, G. K. (1964).
Biochemistry, N.Y. 3, 723.

ABDERHALDEN, R. (1961).
Clinical Enzymology. London: D. Van. Nostrand Company Inc.

ADDIS, T. (1949).
Proc.nat.Acad.Sci. U.S.A. 35, 194.

ALBERT-RECHT, F. (1959).
Clinica chim. Acta, 4, 627.

ALTMAN, K.A. and STELLATE, R. (1963).
Clin.Chem. 9, 63.

ANDERSON, A. J. and MACLAGAN, N. F. (1955).
Biochem.J. 59, 638.

ANDERSON, S. B. (1963).
Protides of the Biological Fluids, Ed. H. Peeters. Proceedings of the 10th Colloqium, Bruges, 1962, p.157. Amsterdam: Elsevier.

ANDRES, G. A., MORGAN, C., HSU, K. C., RIFKIND, R. A., and SEEGAL, B. C. (1962).
J.exp.Med. 115, 929.

ANDREWS, P. (1965).
Biochem.J. 96, 595.

APPELLA, E. and MARKERT, C. L. (1961).
Biochem. biophys. Res. Commun. 6, 171.

ARTURSON, G., GRANATH, K., THOREN, L., and WALLENIUS, G. (1964).
Acta. chir.scand. 127, 543.

ARTURSON, G. and WALLENIUS, G. (1964a).
Scand. J. clin. Lab. Invest. 16, 76.

ARTURSON, G. and WALLENIUS, G. (1964b).
Scand. J. clin. Lab. Invest. 16, 81.

ASSCHER, A. W. and JONES, J. H. (1965).
Postgrad. med. J. 41, 425.

BARCELO, R. and POLLACK, V. E. (1966).
Can. med. Ass. J. 94, 269.

BAYLISS, L. E. and LUNDSGAARD, E. (1932).
J. Physiol., Lond. 74, 279.

BAYLISS, L. E., KERRIDGE, T. M. T. and RUSSELL, D. S. (1933).
J. Physiol., Lond. 77, 386.

BECKER, E. L. (1964).
Renal Metabolism and Epidemiology of Some Renal Diseases, Ed. J. Metcoff.
Proceedings of the 15th annual conference on the kidney, p.207.
New York: National Kidney Foundation.

BELL, E. T. (1929).
Am. J. Path. 5, 587.

BELL, E. T. (1938).
Am. J. Path. 14, 691.

BERGGARD, I. (1961a).
Clinica chim. Acta, 6, 413.

BERGGARD, I. (1961b).
Clinica chim. Acta, 6, 545.

BERGGARD, I., CLEVE, H. and BEARN, A. G. (1964).
Clinica chim. Acta, 10, 1.

BERGLUND, H., SCRIVER, W. and MEDES, G. (1935).
Proteinuria and plasma proteins in The Kidney in Health and Disease,
Ed. H. Berglund and G. Medes. London: Henry Kimpton.

BERLYNE, G. M., VARLEY, H., NILWARANGKUR, S. and HOERNI, M. (1964).
Lancet, ii, 874.

BICKFORD, R. G. and WINTON, F. R. (1937).
J.Physiol., Lond. 89, 198.

BIETER, R. N. (1931).
J. Pharmac. exp. Ther. 43, 407.

BLAINEY, J. D., BREWER, D. B., HARDWICKE, J. and SOOTHILL, J. F. (1960).
Q. Jl. Med. 53, 235.

BOYCE, W. H. and KING, J. S. (1960).
Biology of Pyelonephritis, Ed. E. L. Quinn and E. H. Kass.
Henry Ford Hospital International Symposium, p.551. London: J. & A. Churchill.

BOYCE, W. H., KING, J. S. and FIELDEN, M. L. (1961).
J. clin. Invest. 40, 1453.

BOYCE, W. H., KING, J. S., LITTLE, J. M. and ARTOM, C. (1958).
J. clin. Invest. 37, 1658.

BOYCE, W. H., GARVEY, F. K. and NORFLEET, C. M. (1954).
J. clin. Invest. 33, 1287.

BOTT, P. A. and RICHARDS, A. N. (1941).
J. biol. Chem. 141, 291.

BREWER, D. B. (1951).
Proc. R. Soc. Med. 44, 561.

BROCH, O. J. and BRODWALL, E. (1958).
Acta med. scand. 160, 353.

CAMERON, J. S. (1966).
Proc. R. Soc. Med. 59, 512.

CAMERON, J. S. and BLANDFORD, G. (1966).
Lancet, ii, 242.

CAMERON, J. S. and WHITE, R. H. R. (1965).
Lancet, i, 463.

CARAWAY, W. T. (1960).
Microchemical Methods for Blood Analysis, p.98.
Springfield, Illinois: C. C. Thomas.

CARGILL, W. H. and BRUNER, H. D. (1951).
J. Pharmac. exp. Ther. 103, 339.

CHARLWOOD, P. A. (1963).
Biochem. J. 88, 394.

CHINARD, F. P., LAUSON, H. D., EDER, H. A., GRIEF, R. L. and HILLER, A. (1954).
J. clin. Invest. 33, 621.

CHINSKY, M., SHMAGRANOFF, G. L. and SHERRY, S. (1956).
J. Lab. clin. Med. 47, 108.

CHRISTIANSEN, J. A., JENSON, C. E. and MARCKER, K. (1957).
Acta. chem. scand. 11, 402.

CHURG, J., MAUTNER, W., GRISHMAN, E. and EISNER, G. M. (1962).
Archs. intern. Med. 109, 97.

COLTORI, M., ASCIONE, A., GIUSTI, G. and DI SIMONE, A. (1962).
Rif. med. 76, 1313.

CORNILLOT, P., BOURILLON, R., MICHON, J. and GOT, R. (1963).
Biochim. biophys. Acta. 71, 89.

CROCKSON, R. A. (1961).
Lancet, i, 139.

CUSHNY, A. R. (1926).
The Secretion of Urine, 2nd ed. London: Longmans, Green & Co.

DE HAAN, J. (1922).
J. Physiol., Lond. 56, 444.

DI FERRANTE, N. (1957).
J. clin. Invest. 36, 1516.

DIRKS, J. H., CLAPP, J. R. and BERLINER, R. W. (1964).
J. clin. Invest. 43, 916.

DISCHE, R. M., PAPS, G. D., GRAUER, A. and DISCHE, Z. (1965).
Biochem. biophys. Res. Commun. 20, 63.

DIXON, M. and WEBB, E. C. (1964).
The Enzymes, 2nd ed. London: Longmans, Green & Co. Ltd.

DOCK, W. (1942).
New Engl. J. Med. 227, 633.

DRUMMOND, K. N., MICHAEL, A. F., GOOD, R. A. and VERNIER, R. L. (1966).
J. clin. Invest. 45, 620.

DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. and SMITH, F. (1956).
Analyt. Chem. 28, 350.

EDWARDS, K., JEPSON, R. P. and WOOD, K. F. (1960).
Br. med. J. i, 30.

ELLIASCH, H., SELLERS, A. L., ROSENFELD, S. and MARMORSTON, J. (1955).
J. exp. Med. 101, 129.

EMSLIE-SMITH, D., JOHNSTONE, J. H., THOMSON, M. B. and LOWE, K. G. (1956).
Clin. Sci. 15, 171.

FAHEY, J. L. (1962).
Adv. Immun. 2, 42.

FAHEY, J. L. (1963).
J. Immun. 91, 439.

FAILING, J. F., BUCKLEY, M. W. and ZAK, B. (1960).
J. clin. Path. 33, 83.

FARQUHAR, M. G. (1959).
Proceedings of the 10th Annual Conference on the Nephrotic Syndrome,
Ed. J. Metcoff, p.2. New York, 1958: National Kidney Disease Foundation.

FARQUHAR, M. G. (1960).
J. biophys. biochem. Cytol. 7, 297.

FARQUHAR, M. G., WISSIG, S. L. and PALADE, G. E. (1961).
J. exp. Med. 113, 47.

FINE, J. (1965).
Br. med. J. i, 1209.

FISHER, E. R. and HELLSTROM, H. R. (1962).
Lab. Invest. 11, 617.

FLODIN, P. (1962).
Dextran gels and their Applications in Gel Filtration.
Uppsala: Pharmacia.

FOLLI, G., POLLAK, V. E., REID, R. T. W., PIRANI, C. L. and KARK, R. M. (1958).
Ann. intern. Med. 49, 775.

FRANKLIN, E. C. and KUNKEL, H. G. (1957).
J. clin. Invest. 38, 2159.

FRAZER, S. C. (1958).
Br. Med. J. i, 981.

FREEDMAN, P., PETERS, J. H. and KARK, R. M. (1960).
Ann. intern. Med. 105, 524.

FREEMAN, T. and JOEKES, A. M. (1957).
Acta. med. Scand. 157, 43.

GALAN, E. and MASO, C. (1957).
Pediatrics, Springfield, 20, 610.

GARDNER, K. D. (1961).
J. clin. Invest. 40, 525.

GELL, P. G. H. (1957).
J. clin. Path. 10, 67.

GILSON, S. B. (1949).
Proc. soc. exp. Biol. Med. (N.Y.) 72, 608.

GITLIN, D. and JANEWAY, C. A. (1952).
J. clin. Invest. 31, 223.

GITLIN, D., JANEWAY, C. A. and FARR, L. E. (1956).
J. clin. Invest. 35, 44.

GOODMAN, H. (1961).
Proceedings of the 12th Annual Conference on the Nephrotic Syndrome,
Ed. J. Metcoff, p.245. New York, 1960: National Kidney Disease Foundation.

GOODMAN, H. C. and BAXTER, J. H. (1956).
Proc. Soc. exp. Biol. Med. (N.Y.) 93, 136.

GORNALL, A. G., BARDAWILL, C. J. and DAVID, M. D. (1949).
J. biol. Chem. 177, 751.

GRABAR, P. and BURTIN, P. (1964).
Immunoelectrophoretic Analysis, London: Elsevier.

GRANATH, K. A. and FLODIN, P. (1961).
Makromolek. Chem. 48, 160.

GRANT, G. H. (1957).
J. clin. Path. 10, 360.

GRANT, G. H. (1959).
J. clin. Path. 12, 510.

GRAY, I. (1953).
Am. J. Physiol. 174, 462.

GRAY, I., SITTERI, P. K., and PULASKI, E. J. (1951).
Proc. Soc. exp. Biol. Med. (N.Y.) 77, 626.

GREGOIRE, F., MALMENDIER, C. and LAMBERT, P. P. (1958).
Am. J. Med. 25, 516.

GRIEBLE, H. G., COURCON, J. and GRABAR, P. (1965).
J. Lab. clin. Med. 66, 216.

GRONWALL, A. (1957).
Dextran and its use in Colloidal Infusion Solutions.
Oxford: Blackwell.

HALL, B. V. (1957).
Am. Heart J. 54, 1.

HALL, C. E. and SLAYTER, H. S. (1959).
J. biophys. biochem. Cytol. 5, 11.

HALWER, M., NUTTING, G. C. and BRICE, B. A. (1951).
J. Am. chem. Soc. 73, 2786.

HANLEY, W. B. (1964).
Br. med. J. i, 936.

HANLEY, W. B., BOYER, S. H. and NAUGHTON, M. A. (1966).
Nature, Lond. 209, 996.

HARDWICKE, J. (1965).
Clinica chim. Acta. 12, 89.

HARDWICKE, J. and SOOTHILL, J. F. (1961).
Glomerular damage in terms of "pore size".
Ciba Foundation Symposium on Renal Biopsy, Ed. G. E. W. Wolstenholme and M. P. Cameron. London: J. & A. Churchill.

HARDWICKE, J. and SQUIRE, J. (1955).
Clin. Sci. 14, 509.

HARDWICKE, J. and ST. CYR, C.H. de V. (1961).
Clinica chim. Acta. 6, 503.

HARMS, W. S., BLITCH, P. G., JOHNSON, A. C. and FINDLEY, T. (1962).
Metabolism, 11, 542.

HEREMANS, J. F., HEREMANS, M.-Th. and SCHULTZE, H. E. (1959).
Clinica chim. Acta. 4, 96.

HEYMANN, W., GILKEY, C. and LEWIS, M. (1956).
Am. J. Dis. Child. 91, 570.

HILLER, A., GREIF, R. L. and BECKMAN, W. W. (1948).
J. biol. Chem. 176, 1421.

HITZIG, W. H., AURRICCHIO, S. and BENNINGER, J. H. (1965).
Klin. Wschr. 43, 1154.

HOWE, A. F., GROOM, T. and CARTER, R. G. (1964).
Analyt. Biochem. 9, 443.

HUGHES, W. L. (1957).
Proceedings of the 8th Annual Conference on the Nephrotic Syndrome,
Ed. J. Metcoff, p.22. New York, 1956: National Nephrosis Foundation, Inc.

HULME, B., and HARDWICKE, J. (1966).
Proc. R. Soc. Med. 59, 509.

HUNT, J. N. (1948).
Biochem. J. 42, 104.

ISLIKER, H. C. (1957).
Adv. Protein Chem. 12, 388.

ITZHAKI, R. F. and GILL, D. M. (1964).
Analyt. Biochem. 9, 401.

JAMES, J. A. and ASHWORTH, C. T. (1961).
Am. J. Path. 38, 515.

JAMES, J., GORDEILO, G. and METCOFF, J. (1956).
J. clin. Invest. 33, 1346.

JESTING, E. (1964).
Scand. J. clin. Lab. Invest. 16, 549.

JOACHIM, G. R., CAMERON, J. S., SCHWARTZ, M. and BECKER, E. L. (1964).
J. clin. Invest. 43, 2332.

KAITZ, A. L. (1959).
J. Lab. clin. Med. 53, 186.

KAO, K. T., HIZER, C. A., DAWSON, R. L. and McGAVACK, T. H. (1965).
Proc. Soc. exp. Biol. Med. (N.Y.) 119, 193.

KARK, R. M., PIRANI, C. L., POLLAK, C. E., MUEHRCKE, C. and BLAINEY, J. D. (1958).
Ann. intern. Med. 49, 751.

KARMEN, A. (1955).
J. clin. Invest. 34, 131.

KASANEN, A. and HARRI, J. (1961).
Annls.Med. intern. Fenn. 50, 19.

KEMP, E. and LAURSENT, T. (1960).
Scand.J. clin. Lab. Invest. 12, 463.

KILLANDER, J. and FLODIN, P. (1962).
Biochim. Biophys. Acta. 63, 403.
KING, S.E. (1959). New York State J. Med. 59, 825.
KING, J. S. and BOYCE, W. H. (1963).
High Molecular Weight Substances in Human Urine.
Springfield, Illinois: C. C. Thomas.

KLUTSCH, K., HEIDLAND, A. and KAMMERER, H. (1965).
Medsche. Klin. 60, 464.

KOECHLIN, B. A. (1952).
J. Am. chem. Soc. 74, 2649.

KRAMER, B., STERN, K. and HELLMAN, L. (1957).
Proceedings of the 8th Annual Conference on the Nephrotic Syndrome,
Ed. J. Metcoff, p.30. New York, 1956: National Nephrosis Foundation, Inc.

KURTZ, S. M. and McMANUS, J. F. A. (1959).
Am. Heart. J. 58, 357.

LAMBERT, P. P. and GREGOIRE, F. (1955).
Archs. int. Physiol. Biochim. 63, 7.

LANDIS, E. M. (1946).
Ann. N. Y. Acad. Sci. 46, 713.

LATHEM, W. (1959).
J. clin. Invest. 38,652.

LAURENT, B. (1958).
Scand. J. clin. Lab. Invest. Suppl. 32.

LAURENT, T. C. and KILLANDER, J. (1964).
J. Chromat. 14, 317.

LAUSON, H. D., FORMAN, C. W., McNAMARA, H., MATTAR, G. and BARNETT, H. L. (1954).
J. clin. Invest. 33, 657.

LEWIS, L. A. (1963).
Biochem. Clinics 2, 25.

LICHTY, J. A., HAVILL, W. H. and WHIPPLE, G. W. (1932).
J. exp. Med. 55, 603.

LINDGREN, F. T. and NICOLS, A. V. (1960).
Structure and function of human serum lipoproteins in The Plasma Proteins, Vol. II, Ed. F. W. Putnam. London: Academic Press.

LIPMANN, R. W. (1957).
Urine and the Urinary Sediment: A practical manual and atlas, 2nd ed. Springfield, Illinois: C. C. Thomas.

LOWGREN, E. (1955).
Acta. med. scand. Suppl. 300.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951).
J. biol. Chem. 193, 265.

MACDONALD, M. K., LAMBIE, A. T. and ROBSON, J. S. (1959).
Scott. med. J. 4, 415.

MACKENZIE, D. H. (1953).
Br. J. exp. Path. 34, 596.

MAIORCA, R. and SCARPIONI, L. (1963).
Clinica chim. Acta, 8, 710.

MALAMOS, B., DONTAS, A. S., KOUTRAS, D. A., MARKETOS, S., SFONTOURIS, J. and PAPANICOLAOU, N. (1966).
Lancet, i, 943.

MALMENDIER, C., DE KOSTER, K. P. and LAMBERT, P. P. (1960).
Clin. Sci. 19, 605.

MARSH, W. H., FINGERHUT, B. and MILLER, H. (1965).
Clin. Chem. 11, 624.

MARSHALL, M. E. and DEUTSCH, H. F. (1950).
Am. J. Physiol. 163, 461.

MARTINEZ-CARRION, M., RIVA, F., TURANO, C. and FASELLA, P. (1965).
Biochem. biophys. Res. Commun. 20, 206.

MANDL, R. H. (1962).
Protides of the Biological Fluids, Ed. H. Peeters.
Proceedings of the 9th Colloquium, Bruges, 1961, p.85. Amsterdam: Elsevier.

MAUNSBACH, A. B., MADDEN, S. C. and LATTA, H. (1962).
Lab. Invest. 11, 421.

MAXFIELD, M. (1961).
Biochim. biophys. Acta, 49, 548.

MAYCOCK, W. D'A. and RICKETTS, C. R. (1961).
Nature, Lond. 192, 174.

MAYERSON, H. S., WOLFRAM, C. G., SHIRLEY, H. H., and WASSERMAN, K. (1960).
Am. J. Physiol. 198, 155.

McCRORY, W. W., RAPOPORT, M. and FLEISCHER, D. S. (1959).
Pediatrics, Springfield, 23, 861.

McGARRY, E., SEHON, A. H. and ROSE, B. (1955).
J. clin. Invest. 34, 832.

McGREGOR, L. (1929a).
Am. J. Path. 5, 545.

McGREGOR, L. (1929b).
Am. J. Path. 5, 559.

MENDEL, D. (1961).
J. Physiol., Lond. 156, 544.

MICHAEL, A. F., DRUMMOND, K. N., GOOD, R. A. and VERNIER, R. L. (1964).
J. clin. Invest. 43, 1291.

MILLIEZ, P., HARTMANN, L. and LAGRUE, G. (1959).
J. Urol. med. chir. 65, 248.

MIRSKY, I. A., BLOCK, S., OSHER, S. and BROH-KAHN, R. (1948).
J. clin. Invest. 27, 818.

MIYASATO, F. and POLLAK, V. E. (1966).
J. Lab. clin. Med. 67, 1036.

MONKE, J. V. and YUILE, C. L. (1940).
J. exp. Med. 72, 149.

MONTGOMERY, G. L. (1965).
Textbook of Pathology. Edinburgh and London: E. & S. Livingstone.

MORONEY, M. J. (1963).
Facts from Figures, 3rd ed. Harmondsworth: Penguin Books Ltd.

MORRIS, J. O. R. (1964).
J. Chromat. 16, 167.

MORRISON, A. B. and GARDNER, K. D. (1963).
J. exp. Med. 118, 479.

MOVAT, H. Z., STEINER, J. W. and SLATER, R. J. (1961).
The fine structure of the glomerulus in Bright's disease: a clinico-pathological study. Ciba Foundation Symposium on Renal Biopsy, Ed. G. E. W. Wolstenholme and M. P. Cameron. London: J. & A. Churchill.

MUEHRKE, R. C., KARK, R. M., PIRANI, C. L. and POLLAK, V. E. (1957).
Medicine, Baltimore, 36, 1.

MUELLER, C. B. (1958).
Am. Heart J. 55, 304.

NEALE, F. C. (1955).
J. clin. Path. 8, 334.

NETTLESHIP, A., STROTHER, J. A. and SMITH, H. S. (1963).
Clin. Chem. 9, 608.

NICHOLSON, T. F. (1949).
Biochem. J. 45, 112.

NILEHN, J. E. and HILSSON, I. M. (1964).
Scand. J. Haematol. 1, 313.

NORBY, S. (1964).
Expl. Cell Res. 36, 663.

NUSSLE, D. and ROYER, P. (1964).
Protides of the Biological Fluids, Ed. H. Peeters. Proceedings of the 11th Colloquium, Bruges, 1963, p.220. Amsterdam: Elsevier.

OKUDA, R., KAPLAN, M. H, CUPPAGE, F. E. and HEYMANN, W. (1965).
J. Lab. clin. Med. 66, 204.

OLIVER, J. (1948).
J. Mt. Sinai Hosp. 15, 175.

OLIVER, J. (1950).
Am. J. Med. 9, 88.

OLIVER, J., MACDOWELL, M. and LEE, Y. C. (1954).
J. exp. Med. 99, 589.

ONCLEY, J. L., SCATCHARD, G. & BROWN, A. (1947).
J. Phys. Chem. 51, 184.

OSMAN, A. A. (1937).
Original papers of Richard Bright on Renal disease. Oxford: University Press.

OUCHTERLONY, O. (1962).
Prog. Allergy, 6, 30.

OZAWA, Y. and YAMUCHI, M. (1963).
Keio J. Med. 12, 37.

PAPPENHEIMER, J. R. (1953).
Physiol. Rev. 33, 387.

PAPPENHEIMER, J. R., RENKIN, E. M. and BORRERO, L. (1951).
Am. J. Physiol. 167, 13.

PARVIN, R., PANDE, S. V. and VENKITASUBRAMANIAN, T. A. (1965).
Analyt. Biochem. 12, 219.

PATERSON, W. G. (1966).
Personal communication.

PATTE, J. C., BALDASSAIRE, G. and LORET, J. (1958).
Revue fr. Etud. clin. biol. 3, 960.

PEASE, D. C. and BAKER, K. F. (1950).
Am. J. Anat. (1950).

PETERS, J. P. and VAN SLYKE, D. D. (1932).
Quantitative Clinical Chemistry, Vol. II, p. 381.
London: Bailliere, Tindall and Cox.

PEETOOM, F. (1963).
The Agar Precipitation Technique and its Application as a Diagnostic and Analytical Method. Edinburgh: Oliver & Boyd.

PHELPS, R. A. and PUTNAM, F. W. (1960).
Chemical composition and molecular parameters of purified plasma proteins in The Plasma Proteins, Vol. I, Ed. F. W. Putnam. London: Academic Press.

POPENHOE, E. A. (1955).
J. biol. Chem. 217, 61.

PORTER, P. (1964).
J. comp. Path. Ther. 74, 108.

RATHER, L. J. (1952).
Medicine, Baltimore, 31, 357.

REVILLARD, J. (1964).
Les Proteinuries au cours des Malades Renales.
Interne "Medaille d'Or" des Hospitaux de Lyon.

RICKETTS, C. R. (1966).
Nature, Lond., 210, 1113.

RICKETTS, C. R., COX, J. S. G., FITZMAURICE, C. and MOSS, G. F. (1965).
Nature, Lond., 208, 37.

RIGAS, D. A. and HELLER, C. G. (1951).
J. clin. Invest. 30, 853.

ROBINSON, J. R. (1962).
Principles of renal physiology in Renal Disease, Ed. D. A. K. Black.
Oxford: Blackwell.

ROBINSON, R. R., ASHWORTH, C. T., GLOVER, S. N., PHILLIPPI, P. J., LECOCQ, F. R. and LANGELIER, P. R. (1961a).
Am. J. Path. 39, 291.

ROBINSON, R. R., ASHWORTH, C. T., GLOVER, S. N., PHILLIPPI, P. J., LECOCQ, F. R. and LANGELIER, P. R. (1961b).
Am. J. Path. 39, 405.

ROSALKI, S. B. and WILKINSON, J. H. (1959).
Lancet, ii, 327.

ROSENFELD, E. L. and LUKOMSKAYA, J. S. (1957).
Clinica chim. Acta, 2, 105.

ROSENFELD, E. L. and SAIENKO, A. S. (1964).
Clinica chim. Acta, 10, 223.

ROWE, D. S. (1957).
Biochem. J. 67, 435.

ROWE, D. S. and SOOTHILL, J. F. (1961a).
Clin. Sci. 21, 75.

ROWE, D. S. and SOOTHILL, J. F. (1961b).
Clin. Sci. 21, 87.

RYLE, A. P. (1965).
Nature, Lond., 206, 1256.

SAIFER, A. and GERSTENFELD, S. (1964).
Clin. Chem. 10, 321.

SAXON, E. I., HINKLEY, W. C., VOGEL, W. C., ZEIVE, L. (1957).
Archs. intern. Med. 99, 607.

SCHEIDEGGER, J. J. (1955).
Int. Archs. Allergy appl. Immun. 7, 103.

SCHERAGA, H. A. and LASKOWSKI, M. (1957).
Adv. Protein Chem. 12, 1.

SCHULTZE, H. E. (1957).
Scand. J. clin. lab. Invest. Suppl. 31.

SCHULTZE, H. E. (1962).
Archs. Biochem. Biophys. Suppl. 1.

SCHULTZE, H. E., GOLLNER, J., HEIDE, K., SCHONENBERGER, M. S. and SCHWICK, G. S. (1955).
2. Naturf. 10b, 463.

SELLERS, A. L., GRIGGS, N., MARMORSTON, J. and GOODMAN, H. C. (1954).
J. exp. Med. 100, 1.

SELLERS, A. L. and MARMORSTON, J. (1956).
J. Lab. clin. Med. 47, 248.

SEMPLE, R. E. (1957).
Can. J. Biochem. Physiol. 35, 383.

SEPHAHA, G. C., BHANDARI, C. R., and VIJAYVARGIYA, N. (1961).
Indian J. med. Res. 49, 68.

SHIM, B. and BEARN, A. (1964).
J. exp. Med. 120, 611.

SHORE, B. (1957).
Archs. Biochem. Biophys. 71, 1.

SHUSTER, S., JONES, J. H. and FLYNN, G. (1963).
Br. J. exp. Path. 14, 145.

SINGH, A. K. and SHINTON, N. K. (1965).
J. clin. Path. 18, 349.

SLATER, R. J., O'DOHERTY, N. J. and DE WOLFE, M. S. (1960).
Pediatrics, N. Y., 26, 190.

SMETANA, H. (1947).
Am. J. Path. 23, 255.

SMITH, I. (1960).
Chromatographic and Electrophoretic Techniques, Vol. II Zone Electrophoresis.
London: Heinmann.

SMITH, E. L., BROWN, D. M., WEIMER, H. E. and WINZLER, R. J. (1950).
J. biol. Chem. 185, 569.

SMITH, B. W. and ROE, J. H. (1952).
J. appl. Physiol. 4, 666.

SOBOTKA, H. (1955).
Monomolecular layers: their application in physiology and medicine in
Medical Physics, Ed. O. Glasser. Vol. I. Chicago: Year Book Publishers.

SOOTHILL, J. F. (1962).
J. Lab. clin. Med. 59, 859.

SPECTOR, W. G. (1954a)
J. Path. Bact. 68, 187.

SPECTOR, W. G. (1954b).
Clin. Sci. 13, 1.

SPIRO, D. (1959).
Am. J. Path. 35, 47.

SQUIRE, J. R., BLAINEY, J. D. and HARDWICKE, J. (1957).
Br. med. Bull. 13, 43.

SQUIRE, J. R., HARDWICKE, J. and SOOTHILL, J. F. (1962).
Proteinuria in Renal Disease, Ed. D. A. K. Black. Oxford: Blackwell.

SQUIRE, P. G. (1964).
Archs. Biochem. Biophys. 107, 471.

STEBLAY, R. W. and LEPPER, M. H. (1961).
J. Immun. 87, 636.

STEVENS, D. L., SKEGGS, L. T., CHASSON, A. L., GRADY, H. J., STANLEY, M. A. (1962).
Technicon AutoAnalyzer Methodology, File no. 11. New York: Technicon Instruments Corporation.

SWAHN, B. (1953).
Scand. J. clin. Lab. Invest. Suppl. 9.

SZASZ, G., BARANYAI, P., CZIRBESZ, Z. and CSAKI, P. (1965).
Klin. Wschr. 43, 783.

TAKAHASHI, S. and SCHMID, K. (1962).
Biochim. biophys. Acta. 63, 343.

TATA, J. R. (1959).
Nature, Lond., 183, 877.

TERRY, R., HAWKINS, D. R., CHURCH, E. M., and WHIPPLE, G. H. (1948).
J. exp. Med. 87, 561.

TERRY, R., YUILE, C. L., GOLODETZ, A., PHILIPS, C. E. and WHITE, R. R. (1953).
J. Lab. clin. Med. 42, 6.

TOBIAS, G. J. and McLAUGHLIN, R. F. and HOPPER, J. (1962).
New Eng. J. Med. 266, 317.

URIEL, J. (1964).
The characterization reactions of the protein constituents following electrophoresis or immuno-electrophoresis in agar, in Immunoelectrophoretic Analysis, Ed. P. Grabar and P. Burtin. London: Elsevier.

VAN GOIDSENHOVEN, G., WILKOFF, L. and KIRSNER, J. B. (1958).
Gastroenterology, 34, 421.

VAN LOON, E. J., LIKINS, M. R. and SEGER, A. J. (1952).
Am. J. clin. Path. 22, 1134.

VASSALLI, P., MORRIS, R. H., and McCLUSKEY, R. T. (1963).
J. exp. Med. 118, 467.

VASSALLI, P., SIMON, G., and ROUILLER, C. (1963).
Am. J. Path. 63, 578.

VERE, D. W. and WALDUCK, A. (1966).
Clin. Sci. 30, 315.

VERNIER, R. L. (1961).
Ultrastructure of the glomerulus and changes in fine structure associated with increased permeability of the glomerulus to protein in Ciba Foundation Symposium on Renal Biopsy, Ed. G. E. W. Wolstenholme and M. P. Cameron. London: J. & A. Churchill.

VERNIER, R. L., FARQUHAR, M. G., BRUNSON, J. G. and GOOD, R. A., (1958).
Am. J. Dis. Child. 96, 306.

VIMTRUP, B. (1928).
Am. J. Anat. 41, 123.

WACKER, W. E. C. and DORFMAN, L. E. (1962).
J. Am. med. Ass. 181, 972.

WAKIM, K. G. and MACKENZIE, B. F. (1960).
J. Lab. clin. Med. 55, 510.

WALKER, A. M., BOTT, P. A., OLIVER, J., MACDOWELL, M. C. (1941).
Am. J. Physiol. 134, 580.

WALLENIUS, G. (1954).
Acta Soc. Med. upsal. Suppl. 4.

WARBURG, O. and CHRISTIAN, W. (1941).
Biochem. Z. 310, 384.

de WARDENER, H. E. (1963).
The Kidney, 2nd ed. London: J. & A. Churchill.

WATERHOUSE, C. and HOLLER, J. (1948).
J. clin. Invest. 27, 560.

WATSON, D. (1964).
Clin. Chem. 10, 559.

WEARN, J. T. and RICHARDS, A. N. (1924).
Am. J. Physiol. 71, 209.

WEBB, T. W., ROSE, B. and SEHON, A. H. (1958a).
Can. J. Biochem. Physiol. 36, 1159.

WEBB, T. W., ROSE, B. and SEHON, A. H. (1958b).
Can. J. Biochem. Physiol. 36, 1167.

WHITEHEAD, P. H. (1965).
Biochem. J. 95, 8P.

WILKINSON, J. H. (1962).
An Introduction to Diagnostic Enzymology. London: Edward Arnold.

WILLS, M. R. and McGOWAN, G. K. (1963).
J. clin. Path. 16, 487.

WINZLER, R. H. (1960).
Glycoproteins in The Plasma Proteins, Vol. I, Ed. F. W. Putnam. London: Academic Press.

WOLBACH, S. B. and BLACKFAN, K. D. (1930).
Am. J. med. Sci. 180, 453.

WOLVIUS, D., and VERSCHURE, J. C. M. (1957).
J. clin. Path. 10, 80.

WROBLEWSKI, F. and LA DUE, J. S. (1955).
Proc. Soc. exp. Biol. Med. (N.Y.) 90, 210.

ZETTERGREN, L. (1962).
Acta chir. Scand. 123, 439.